STUDIES ON ALLOPREGNANE-3α,20α-DIOL IN HUMAN URINES.

by

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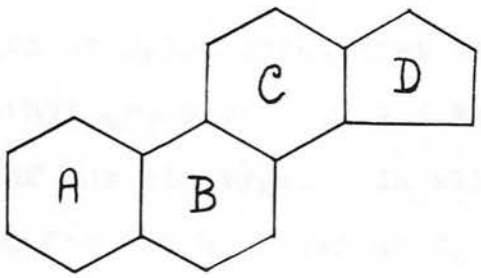
GENERAL INTRODUCTION

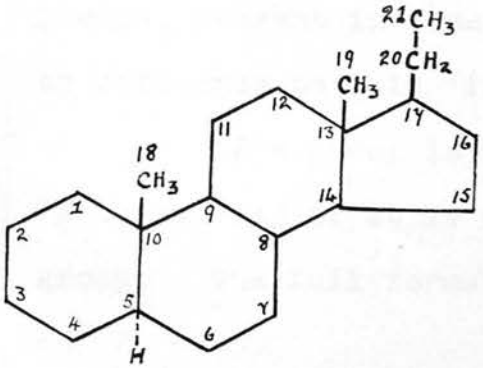
(a) Steric relationships of pregnane and allopregnane derivatives.

The name 'steroid' is reserved as a group name for all substances containing the 1,2 cyclopentenophenanthrene carbon skeleton. This structure and the system used for differentiating the various rings is shown below (I).

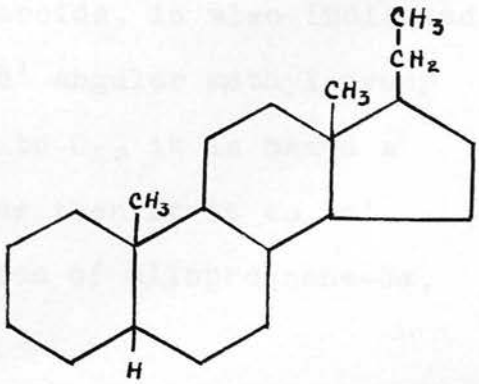


(I)

The parent hydrocarbon of the compound studied is, however, allopregnane (II). The formula of pregnane (III) is given to allow direct comparison. The system of numbering used is indicated in (II).



(II) Allopregnane

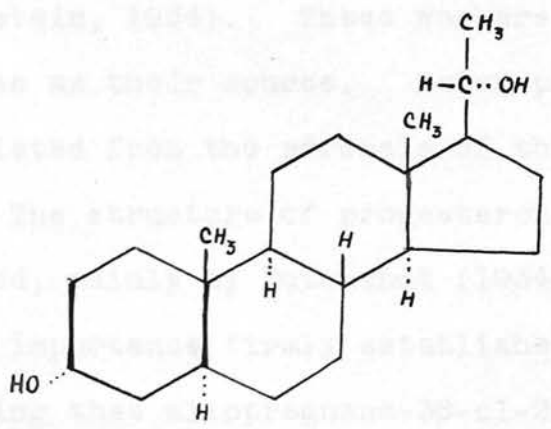


(III) Pregnane

These two hydrocarbons differ only in the relative orientation of rings A and B. In pregnane the orientation is of the cis-decalin type while in allopregnane it is of the trans-decalin type. By convention the angular methyl group attached to C_{10} is considered to be fixed; reference to this fixed group enables relative orientations to be simply, but unequivocally, indicated. In pregnane derivatives, therefore, the provision of a full bond for the hydrogen at C_5 indicates that it is cis to the angular methyl group at C_{10}, and therefore the ring fusion is of the cis type. In allopregnane the dotted bond for the hydrogen at C_5 indicates a trans relationship to C_{18} and therefore the A/B ring fusion is of the trans type. The orientation of rings B/C and of rings C/D is believed to be of the trans-decalin type in all the naturally-occurring steroids which have been isolated.

The orientation of the monovalent functional groups, present in some steroids, is also indicated by reference to this 'fixed' angular methyl group at C_{10}; if a group is cis to C_{18} it is named a 'β' group and if it is trans then it is an 'α' group. The full formulation of allopregnane-3α, 20α /

20α-diol (IV) is therefore:



(IV) Allopregnane-3α,20α-diol.

Short Discussion of Previous Work.

In 1929 Marrian isolated a solid dihydroxy alcohol from the urine of pregnant women. The structure of this alcohol and its relationship to cholesterol and the bile acids was soon established, mainly by Butenandt (1930, 1931). This structure, with some minor modifications mainly due to the later revision of the then accepted ring structure, is that of pregnane-3α,20α-diol.

In 1934, after several years of deliberate searching by many groups of workers, the active hormone progesterone ($\triangle^4$pregnene-3,20-dione) was isolated in a chemically pure condition by several research teams almost simultaneously (Butenandt, Westphal/

Westphal and Hohlweg, 1934; Slotta, Rushig and Fels, 1934; Allen and Wintersteiner, 1934; Hartmann and Wettstein, 1934). These workers had used corpora lutea as their source. Later progesterone was also isolated from the adrenals of the ox (Beall, 1938). The structure of progesterone was soon elucidated, mainly by Butenandt (1934), and its biochemical importance firmly established. It is interesting that allopregnane-3β-ol-20-one is invariably found, with the progesterone, in both the corpus luteum (Hartmann and Wettstein, 1934) and the adrenals (Beall, 1938).

The close chemical relationship of the previously isolated pregnane-3α,20α-diol to progesterone was immediately realised and the fact that it may arise in the body by the reductive metabolism of its progesterone was quite clear. Hartmann and Locher (1935) added to the possibility of reductive metabolism of progesterone when they isolated from human pregnancy urine another solid dihydroxy alcohol, which they realised was a stereoisomer of pregnane-3α,20α-diol, and correctly formulated it as an allopregnanediol, now known to be allopregnane-3α,20α-diol.

One further dihydroxy alcohol, also a stereoisomer of pregnane-3α,20α-diol, was isolated from human/

human pregnancy urine, and recognized as allopregnane-3β,20α-diol in 1938 (Marker and coworkers, 1938a). This diol also bears a close chemical relationship to progesterone, and an even closer relationship to the allopregnane-3β-ol-20-one which is always found with the progesterone.

The remaining stereoisomer of this quartet, pregnane-3β,20α-diol, has never been isolated from normal urinary source.

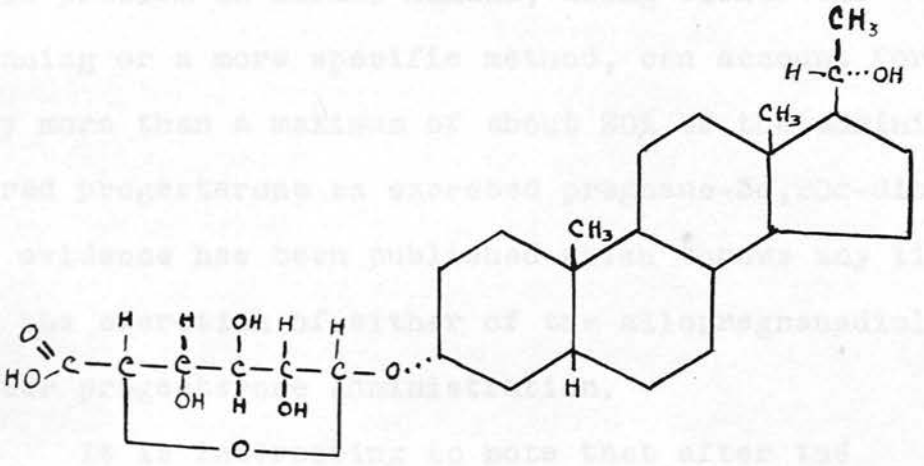
The probability of these three isomeric diols arising from the one source seems, because of their close chemical relationship, to be very high. That this source may be progesterone and that they arise by the metabolic reduction of the $\triangle^4$ double bond and the two ketone groups, seems equally likely. This possibility has been increased by the isolation from urinary sources of several steroids which are, chemically, reduction products of progesterone yet not as fully reduced as the three diols.

It is known that progesterone is released in increasing amounts from the corpus luteum and probably other sites as pregnancy continues, yet progesterone has never been isolated from urinary sources. Marker et al (1937b) worked up some 10,000 gallons of human pregnancy urine in a deliberate search for progesterone but failed to find any. It is/

is notable that the excretion of pregnane-3α,20α-diol in the urine increases markedly as pregnancy progresses. It has not, however, been shown that the urinary excretion of either allopregnane-3α,20α-diol or allopregnane-3β,20α-diol increases during pregnancy.

In 1936 Odell and Marrian showed that pregnane-3α,20α-diol was present in urine as an acid hydrolysable complex. Venning and Browne (1936) showed that this complex was a monoglucuronidate and devised a method of isolation. Venning's method involved extraction of the complex from an almost neutral aqueous phase by n-butanol, in which the glucuronidate is readily soluble, washing of the butanol extract with caustic soda solution to remove acids and phenols, including the oestrogens, and evaporation of the butanol after washing it with water to remove any residual caustic soda. Crystallization could be induced in, and much coloured material removed from, the residue by dissolving it in water or aqueous acetone, and adding dry acetone until the liquor contained 95%(v/v) acetone. Repetition of this acetone precipitation gave a white crystalline sample of 'sodium pregnanediol glucuronidate'. By standardizing extraction conditions Venning (1937) placed this isolation on a quantitative basis. The point of attachment of the glucuronic acid has been established/

established by Heard, Hoffman and Mack (1944) as being at C_3. Heubner and his associates (1944) have prepared pregnane-3α,20α-diol-3β d-glucuronic acid (V) by partial synthesis and shown it to be identical with the natural product.



(V) Pregnane-3α,20α-diol-3βd-glucuronic acid.

Although it appears likely that both allopregnane-3α,20α-diol and allopregnane-3β,20α-diol may be excreted into the urine as a glucuronidate, no evidence has yet been adduced to prove this.

Venning (1937) applied her quantitative method for the estimation of 'pregnanediol' to the problem of the metabolism of progesterone. She was able to show that there was a marked and significant rise in the excretion of 'sodium pregnanediol glucuronidate', and hence of pregnane-3α,20α-diol, after the administration of progesterone to a human patient. This observation/

observation has received ample confirmation from many workers (Sommerville, 1948). Hence progesterone is definitely a precursor of pregnane-3α,20α-diol. It is noteworthy that neither Venning nor any of the many investigators who have subsequently examined this problem in normal humans, using either the Venning or a more specific method, can account for any more than a maximum of about 20% of the administered progesterone as excreted pregnane-3α,20α-diol. No evidence has been published which throws any light on the excretion of either of the allopregnanediols after progesterone administration.

It is interesting to note that after the administration of free pregnane-3α,20α-diol,an accepted 'end-product' of progesterone metabolism, only a fraction of the administered dose can be recovered in the urine. It is stated (Fieser and Fieser, 1949a) that 50% of the dose may be recovered, while Sommerville (1948) claims that only 10-20% can be accounted for. This may well mean that pregnane-3α,20α-diol itself may be further metabolised.

It has been shown recently by Marrian and Gough (1946) and by Marrian and Sutherland (1947) that 'sodium pregnanediol glucuronidate', as isolated/

isolated by Venning's method, is not homogeneous and invariably contains some 20%, by weight, of the glucuronidate of pregnane-3α-ol-20-one. It follows therefore that this pregnanolone is also a metabolite of progesterone. This has been confirmed by Dorfmann, Ross and Shipley (1948) who showed that the excretion of pregnane-3α-ol-20-one is significantly increased after progesterone dosage.

It has, however, been shown convincingly that progesterone is not the only precursor of pregnane-3α,20α-diol. Cuyler and his associates (1940) have shown that the excretion of pregnane-3α,20α-diol rose significantly after they administered deoxycorticosterone ($\triangle^4$-pregnene-21-ol-3,20-dione) to human patients. Bloch (1945) has shown that cholesterol is also a precursor of pregnane-3α,20α-diol. It is not known whether these materials are part of the metabolic pathway involving progesterone and pregnane-3α,20α-diol or not. Again no evidence has been produced to indicate whether any change occurs in the excretion of the two allopregnanediols after administration of such compounds. It is worth noting, however, that as the allo configuration is common to most of the saturated steroids which have so far/

far been isolated and related to the adrenal cortex, it would not be surprising to find that the allo-pregnanediols are metabolic reduction products of a corticoid or alternatively of progesterone in the adrenal cortex. Heard in his chapter on corticoids (1948) considers this a strong possibility.

Although Hartmann and Locher (1935) were the first to isolate allopregnane-3α,20α-diol, the work which led to our knowledge of the quantities present in various urines was performed by Marker and a number of co-workers. This led to the publication of a table (Marker et al., 1938b) indicating the absolute and relative amounts of pregnane-3α,20α-diol, allopregnane-3α,20α-diol and allopregnane-3β, 20α-diol present in a number of urines. This table, which is reproduced on the following page (Table 1) is still quoted unaltered (Pincus and Pearlman, 1943).

Table 1/

Table 1

(expressed in mg./gallon)

Source and species	Pregnane-3α,20α-diol	Allopregnane-3α,20α-diol	Allopregnane-3β,20α-diol
Human pregnancy urine	50	25	6
Human non-pregnancy urine	8	4	-
Mare pregnancy urine	50	25	6
Cow pregnancy urine	25	15	3
Bull urine	100	50	12

Note: Fieser and Fieser(1949[b]) use the same data and source, but record quantities in mg./litre.

On examining the reports on which this table is based, it is difficult to decide how reliable the quoted figures are. Actual isolation of allo-pregnane-3α,20α-diol has only been reported in his studies on the urine from pregnant cows (Marker, ~~et~~ 1938/

1938) and on the urine from bulls (Marker et al, 1938b). In each case this was done by a modification of the method suggested by Hartmann and Locher (1935) in their paper reporting the original isolation of allopregnane-3α,20α-diol. In Marker's method, the urine was hydrolysed and the solids obtained, and after removal of steam volatile material and ketones, the crude diol mixture was acetylated. The diacetates were then separated by fractional crystallization, pregnane-3α,20α-diol diacetate being the least soluble in alcohols, was readily obtained as a crystalline crop. Saponification of both the crystalline crop and the mother liquors gave the free diols, which were then purified by recrystallization from alcohol. Marker et al.(1937a) stated that they isolated both pregnane-3α,20α-diol and allopregnane-3α,20α-diol from the urine of pregnane mares by this method, but although this was their first reported isolation of either of the diols from a natural source, they did not provide experimental details, nor did they state their yields. Marker, however, suggested and used a method of estimating the amounts of the various diols; this involved the oxidation of the crude mixture of diols to the corresponding diones which were then separated by fractional crystallization of their semicarbazones.

The/

The amounts of pregnanedione and allopregnanedione isolated, after hydrolysis of the semicarbazones, provided him with a measure of the amount of the various diols originally present. He lays more stress on such results than on those from direct isolation.

There is no indication in any of his papers of the origin of his quoted figures for the quantities of pregnane-3α,20α-diol and allopregnane-3α,20α-diol in human pregnancy urine. It is notable, also, that the figure quoted in his table (Table 1) for the quantity of allopregnane-3β,20α-diol in human pregnancy urine (6 mg./gall.) did not correspond to the figure of 1-1.5 mg./gall. which he himself had recorded earlier in the same year (Marker *et al*, 1938a). This fourfold difference is recorded without comment.

Marker apparently held the view that the isolation of the three diols, or an indication that they were present from the results of his oxidative procedure on the diol fraction, showed that progesterone was the common and only precursor, and further if progesterone were the precursor, then the/

the relative amounts of the individual diols in the urine would be the same for all species. Marker himself (1939) indicated this when he withdrew his figures for mare's pregnancy urine (see Table 1) and substituted a revised set, saying 'the figures previously reported for mare's pregnancy urine were arbitrarily based on the amount of allopregnane-dione obtained by the oxidation of the total carbinol fraction.....'. But even in this paper, although he isolated allopregnane-3β,20α-diol by precipitation with digitonin before oxidation, he still used the dione method to estimate the quantity of allopregnane-3α,20α-diol present. The revised figures were markedly different from the previous ones. A comparison is given in Table 2.

Table 2.

Steroids in mare's pregnancy urine in mg./gallon.

	Pregnane-3α, 20α-diol	Allopregnane-3α,20α-diol	Allopregnane-3β, 20α-diol
Old values	50	25	6
Revised values	3	2	25

It should be noted that this revision has not been/

been incorporated in tables published since 1939. (See Fieser and Fieser, 1949b; Pincus and Pearlman, 1943).

A further criticism that may be levelled at this work is the fact that very drastic hydrolysis procedures were used on the urines. They involved the addition of sufficient concentrated acid to the boiling urine to give a normal solution and this was boiled for 30 minutes. At a later stage the volatile constituents of the butanol extractable material from the hydrolysed urine were removed by steam distillation from strong KOH; this process must have taken an hour or two. Now it is known that steroids suffer changes and/or decomposition to some extent on boiling with acids. Astwood and Jones (1941) indicate in their graph relating the quantity of pregnane-3α,20α-diol isolable to the period of boiling a standard weight of sodium pregnanediol glucuronidate in N.HCl, that this 30 minute treatment would lead to the loss of some 50% of the available pregnane-3α,20α-diol. It may be noted that a five minute boiling period is said to give maximum results and a ten minute treatment would cause the loss of some 10%. Now it is known that, of the two stereoisomers of decalin, the trans form is the most stable/

stable (Fieser and Fieser, 1949c) it seems therefore very probable that such drastic hydrolysis conditions would lead to the loss of relatively more pregnane-3α,20α-diol than either of the two allo forms. This suggests that both the absolute and the relative amounts of these steroids given by Marker should be viewed with some suspicion.

It seems advisable that the work be repeated.

Part I.

THE INITIAL ISOLATION AND IDENTIFICATION OF ALLOPREGNANE-3α,20α-DIOL.

It has been the practice in this laboratory to retain the filtrates from the isolations of 'sodium pregnanediol glucuronidate' by crystallization from 95% acetone (Venning and Browne, 1936) and filtration at the pump, and evaporate them to dryness. These mother liquor solids were hydrolyzed as batches became large enough by boiling them for ten minutes in a large volume of normal acid. After rapid cooling this hydrolysate was extracted with ether which was washed with normal caustic soda and water. This ether extract was then distilled to dryness giving the neutral ether-soluble extract.

The author received a batch of this neutral ether-soluble material which had arisen from human late pregnancy urine.

Results

This neutral fraction was separated into a ketonic and a non-ketonic fraction by means of a Girard separation (Girard and Sandulesco, 1936).

The non-ketonic fraction was then chromatographed on a column of aluminium oxide and the total benzene/

benzene, ether, acetone and methanol eluates were collected separately.

Recrystallization of the total benzene eluate from dry acetone yielded a good crop of colourless waxy crystals which melted at 59°C. This material was not further examined, but was probably identical with the hydrocarbon which was isolated later from the 3α-hydroxy ketonic fraction (p. 32). A comparison of the two materials by means of a mixed melting-point was performed, but although no depression was obtained, the notorious unreliability of the mixed melting-point technique when applied to saturated hydrocarbons did not allow any conclusions to be drawn.

Recrystallization of the total ether eluate from methanol gave an excellent crop of a crystalline material, recognized by its melting-point and a mixed melting-point with an authentic specimen, as cholesterol which is known to occur in human pregnancy urine (Marker et al., 1938a).

The acetone eluate was rechromatographed and the crystalline solids eluted by benzene containing 10% (v/v) of methanol gave a good yield of allopregnane-3α,20α-diol on recrystallization from methanol. The allopregnane-3α,20α-diol was recognized/

recognized by its melting-point and elementary analysis, by the melting-point and elementary analysis of the diacetate, by the melting-point of the corresponding dione and by the melting-point and nitrogen analysis of the disemicarbazone of this dione.

The methanol eluate was not examined.

The ketonic fraction, which arose from the Girard separation, was separated into a 3α-hydroxy and a 3β-hydroxyketonic fraction by means of digitonin.

The 3α-hydroxyketonic fraction gave, after aluminium oxide chromatography, a further small crop of allopregnane-3α,20α-diol and a sample of a hydrocarbon. The melting-point and elementary analysis of this hydrocarbon corresponded closely to those given by Chibnall and Channon (1929) for n-nonacosane and equally well to those for n-octacosane given by Marker (1938) and were not significantly different from those reported by Hart and Northrup (1935) for n-heptacosane. This hydrocarbon was therefore considered to be a normal paraffin with twenty-seven, twenty-eight or twenty-nine carbon atoms.

The 3β-hydroxyketonic fraction has not been examined.

Experimental/

Experimental

All melting-points recorded in this thesis unless otherwise stated, were determined on an improvised hot-stage melting-point apparatus after the Kofler type (Klyne and Rankeillor, 1947). The thermometer was calibrated by means of pure substances whose melting-points are accurately known. The melting-points are therefore corrected.

Starting material

A dark red gum which was the total neutral ether-soluble fraction isolable after the hydrolysis of the mother liquor from the 95% acetone crystallization of 'sodium pregnanediol glucuronidate'. Laboratory records showed that this had arisen from some 450 litres of human late pregnancy urine.

Girard separation (Girard and Sandulesco, 1936).

Ketones of high molecular weight yield a water-soluble derivative with Girard's reagent 'T' (trimethylammoniumacetohydrazide chloride). Non-ketonic material can then be extracted by non-polar solvent, subsequent hydrolysis of the aqueous phase yields the free ketone which can then also be extracted by non-polar solvent.

The neutral residues (28.6 g.) were dissolved in/

in 300 ml. of dry ethanol containing 31.5 g. of glacial acetic acid. Reagent 'T' (75 g.) was added to the solution and the mixture boiled under reflux for 1 hr. The solution was removed from reflux and chilled rapidly under the tap and finally in chipped ice. Chilled distilled water (100 ml.) containing 18.9 g. NaOH was added and this mixture was poured into a separating funnel containing 1200 ml. of chilled distilled water. The mixture was extracted rapidly once with 600 ml. and thrice with 400 ml. portions of ether. The combined ether extracts were washed once with 250 ml. of 0.5 N.$NaHCO_3$ and three times with 300 ml. portions of distilled water. The ether was then distilled off, leaving 15.4 g. of non-ketonic material.

Sulphuric acid (90 g.) was added slowly to the aqueous phase, after the removal of the non-ketonic material, and the mixture stood at room temperature for 2 hr. The aqueous phase was extracted once with 600 ml. and thrice with 400 ml. portions of ether. The combined ether extracts were washed once with 400 ml. of 0.5 N.$NaHCO_3$ and thrice with 400 ml. portions of distilled water. The ether phase was distilled to dryness, leaving a/

a ketonic fraction weighing 3.6 g.

During this separation a black tarry residue appeared at the ether-water interface. It was allowed to stay in the reaction mixture throughout and was separated off after the last ether extraction. This residue when dry was a black brittle tar weighing 10 g. This was not examined further.

Preliminary separation of the non-ketonic fraction:

The non-ketonic material was separated into four fractions by orthodox elution chromatography on aluminium oxide.

The non-ketonic material (15.4 g.) was dissolved in 450 ml. of sodium dried benzene. Aluminium oxide (450 g. Grade II) was made into a slurry with dry benzene and wet-packed into an orthodox chromatograph column (60 x 5 cm.). The aluminium oxide was graded by Brockmann's method (Brockmann, 1941). The non-ketonic material was poured on to the column and an orthodox chromatograph developed. The eluate was collected in 200 ml. portions in weighed flasks and distilled to dryness on a water bath. The residues were then dried under vacuum at 100°C. and weighed. The eluant was changed whenever two successive 200 ml. portions yielded a fraction weight of less than 5 mg.

Benzene /

Benzene eluate: 2600 ml. of dry benzene were required. Many individual bands of bright colours were developed and eluted. The earlier fractions were mobile yellow oils which did not crystallize; the later fractions were waxy looking solids still yellow in colour.

The total benzene eluate when bulked was a light yellow oil weighing 1.89 g.

Ether eluate: 3000 ml. of dry ether were passed. The bands on the column lost their sharpness and moved slowly down. The earlier fractions had a yellow-green fluorescence although a red band was apparently being eluted. The individual fractions were bulked to give a deep red oil weighing 3.21 g.

Acetone eluate: 4000 ml. of dry acetone were passed. Initially there were no well developed bands on the column but a deep purple band passed rapidly down and was then eluted quite slowly. (This purple band is invariably eluted with pregnane-3α,20α-diol and allopregnane-3α,20α-diol and can be used as a marker for these compounds). The early purple fractions were supersaturated with a crystalline material which separated rapidly after elution.

The elution with acetone was stopped before the/

the fraction weights dropped below 5 mg. as the last four fractions, although weighing from 30-170 mg., were composed of a colourless oil with an aromatic smell which could not be induced to crystallize. Past experience had indicated that acetone can undergo some self-condensation reaction when in contact with Al_2O_3 to give an oily product which was not removed on heating at 100°C. under the vacuum due to a water-pump. Dobriner (1948) has indicated that he has also found this oil formation to be a problem.

The total acetone eluate was a brown crystalline mass of weight 7.3 g.

Methanol eluate: 1600 ml. of methanol were used. This cleaned the column well, only a little brown pigment was not eluted.

Total methanol eluate was a dark brown oil weighing 2.76 g.

Further treatment of the benzene eluate:

The total benzene eluate (1.89 g.) was quite readily dissolved in 10 ml. warm acetone. On cooling a fair crop of crystals appeared and were isolated by filtration. Recrystallization from a small volume of acetone gave 50 mg. of fine white waxy/

waxy looking crystals which when dried melted at 58-59°C. and reset slowly at 58°C.

On mixing with the hydrocarbon isolated from the 3α-ketonic fraction (p. 32), which melted at 62°C., the mixture melted at 59-59.5°C.

The melting-point, appearance and behaviour suggest that this is a hydrocarbon.

The mother liquors were not examined.

Further treatment of the ether eluate:

The total ether eluate (3.21 g.) readily dissolved in 20 ml. warm methanol and deposited fine crystals on standing overnight. Recrystallization of this crop from 20 ml. of ethanol gave 200 mg. of neat white platelets which, when dried, melted at 148.5-149°C.

A mixture with authentic cholesterol (B.D.H.) melted at 148°C. quite sharply.

The material isolated was cholesterol.

No further examination of this fraction was made.

Further treatment of the acetone eluate:

The total acetone eluate (7.30 g.) was dissolved in 200 ml. of 10% (v/v) methanol in benzene and absorbed on to a 220 g. column of Grade II (Brockmann, 1941) Al_2O_3 which had been slurry packed using 10% (v/v) methanol in benzene as the wetting agent/

agent. Elution with the same solvent soon yielded crystalline eluates while much coloured material remained on the column. Table 3 indicates the various fractions obtained.

Table 3

Rechromatograph of total acetone eluate.
Solvent: 10% methanol in benzene.
Fraction volume: 50 ml.

Fraction Nos.	Dry wt.of fraction mg.	Appearance and remarks	M.P.
1-4	5.5	Each fraction weighed less than 2 mg.	oil
5	2.0	Small oil spot	oil
6	33.0	Reddish oil - would not crystallize in acetone	oil
7	175.0	Light brown crystals	207-217°C.
8	437.0	do.	208-210°C.
9	428.0	White crystals	208-211°C.
10	565.0	do.	210-212°C.
11	561.0	do.	210-211°C.
12	602.5	do.	205-207°C.
13	361.0	do.	207-211°C.
14	200.0	do.	210-214°C.
15	74.5	do.	235-236°C.
16	10.0	Brownish oil - would not crystallize from acetone	oil
17	9.0	do. do.	oil
18	3.0	Oil spot	
19	0.2	Nil	

Total weight of material eluted 3.85 g.

Each fraction which had given crystalline material (fractions 7-15) was recrystallized from the minimum volume of methanol. The material was slow to crystallize out (3 days). The mother liquors were bulked and retained (A). The crystals were dried in vacuo over $CaCl_2$ before determining melting-points. Table 4 shows results obtained.

Table 4.

Fraction	Crystalline appearance	M.P.
7	Thick rods, brown	242-244°C.
8	Thick rods + fine needles	223-224°C.
9	Thick rods + sandy crystals	242.5-243°C.
10	Sandy appearance	221-225°C.
11	Sandy appearance + few rods	214-219°C.
12	Sandy appearance	237-239°C.
13	Rods + other material	215-225°C.
14	Clusters of needles	216-219°C.
15	Clusters of needles + few rods	235-237°C.

Fractions 7, 8 and 9 were bulked.
Fractions 10, 11 and 12 were bulked.
Fractions 13, 14 and 15 were bulked.

Each of these bulked fractions was re-crystallized from the minimum volume of methanol, the crops being isolated by filtration and dried over $CaCl_2$ in vacuo. Each yielded bulky glistening rods melting at 243-244°C. Admixture of the various crops with each other did not cause any depression in/

in the melting-point. Careful working up of the mother liquors from this crystallization and A yielded a fair amount of material which melted at temperatures above 240°C.

All the materials which melted above 240°C. were bulked and recrystallized to a constant melting-point of 245-245.5°C. and this on admixture with authentic pregnane-3α,20α-diol (m.p. 236°C.) melted at 214-216°C. Allopregnane-3α,20α-diol melts at 248°C. (Hartmann and Locher, 1935) .

Yield: 220 mg.

4.180 mg. dried over P_2O_5 in vacuo for 6 hr. at 80°C. gave 12.070 mg. CO_2 and 4.130 mg. H_2O.

3.040 mg. similarly dried gave 8.770 mg. CO_2 and 2.960 mg. H_2O.

Found: C, 78.74 and 78.66; H, 11.06 and 10.89.

Calc. for $C_{21}H_{36}O_2$: C, 78.8; H, 11.2%.

Identification of allopregnane-3α,20α-diol.

Preparation of the diacetate

A portion of the material (50 mg.) was refluxed for 1 hr. with 3 ml. dry pyridine and 3 ml. of re-distilled acetic anhydride and allowed to stand overnight. Ice cold water (30 ml.) was added and the crystals which formed were filtered off, washed copiously/

copiously with water, and dried in vacuo over $CaCl_2$ overnight. The solid was washed through the filter paper by a jet of hot methanol and recrystallized from a small volume of methanol. The dry product (48 mg.) melted sharply at 141-142°C.

Allopregnane-3α,20α-diol diacetate melts at 141.5-142.5 (Hartmann and Locher, 1935).

2.800 mg. dried over P_2O_5 for 6 hr. at 80°C. gave 7.688 mg. CO_2 and 2.480 mg. H_2O.

Found: C, 74.57; H, 9.87%.

Calc. for $C_{25}H_{40}O_4$: C, 74.3; H, 9.9%.

A portion of the diacetate (16 mg.) was refluxed with 10 ml. of 98% (v/v) ethanol containing 40 mg. KOH for 2 hr. Most of the alcohol was removed by evaporation under reduced pressure and 30 ml. of water added. The solid was filtered off, copiously washed with water, and dried in vacuo over $CaCl_2$ overnight. The solid was washed through the filter paper with a jet of hot methanol and crystallized from a small volume of methanol. Crystallization was slow but after standing three days in the refrigerator 7.5 mg. of fine rods were isolated and dried in vacuo over $CaCl_2$. This free diol melted at 245-245.5°C. very sharply.

Preparation/

Preparation of the dione

A portion of the diol (35 mg.) was dissolved in 6 ml. of 90% (v/v) acetic acid containing 38 mg. of chromic oxide and allowed to stand at room temperature for 14 hr. Water (30 ml.) was added and the solid filtered off, washed copiously with water, and dried over solid NaOH in vacuo overnight. The solid was washed through the filter paper with a jet of hot methanol and crystallized from aqueous ethanol and recrystallized from a small volume of dry acetone. The product (20 mg.) melted at 200-202°C. with a preliminary softening at 195°C.

Allopregnanedione melts at 204-204.5° (Hartmann and Locher, 1935).

The disemicarbazone was formed from a portion of the dione (15 mg.) by warming it in 3 ml. of pyridine containing 2 drops of water and 45 mg. of semicarbazide hydrochloride and allowing it to stand overnight. Water (20 ml.) was added and the solid filtered off, washed copiously with water, and dried over $CaCl_2$ in vacuo overnight. The solid was washed off the filter paper by a jet of hot methanol and leached well with hot ethanol (the disemicarbazone was too insoluble in the common solvents to be satisfactorily recrystallized).

The/

The material did not melt below 300°C. Allopregnanedione disemicarbazone does not melt below 325°C. (Marker *et al*, 1937a).

Examination of the ketonic fraction.

Separation of the 3α-hydroxyketonic fraction

The ketonic fraction (3.6 g.) was dissolved in 1200 ml. of 90% (v/v) ethanol containing 14.4 g. of digitonin, brought to the boil and allowed to cool slowly overnight. The precipitated 3β-hydroxy digitonide was separated off by centrifugation and the solid washed in the centrifuge tubes with 500 ml. of 90% (v/v) ethanol and then with 500 ml. of dry ether. The solid 3β-hydroxyketonic digitonide was not further examined.

The supernatant liquid and the washings were taken to dryness, dissolved in a small volume of methanol and a large volume of dry ether was added. The free digitonin thus precipitated was filtered off through a fluted filter paper and the filtrate was taken to dryness. This, the 3α-hydroxyketonic fraction, weighed 3.57 g. and was a reddish-brown oil.

Chromatograph/

Chromatograph of the 3α-hydroxyketonic fraction

The whole fraction (3.57 g.) was dissolved in dry benzene and chromatographed through a column of 110 g. Al_2O_3 (Grade II. Brockmann, 1941). Many solid fractions were obtained but only two were thoroughly examined.

The first fraction was eluted by benzene and was the first material to come off the column. The material (160 mg.) crystallized readily from acetone in waxy looking plates which after drying in vacuo over $CaCl_2$ overnight melted at 62°C. Yield, 32 mg. The total yield on heating in vacuo distilled between 140-160°C. under 4 x 10^{-3} mm. of mercury to give a white wax which melted at 62°C. and re-solidified at 61.5°C.

n-Nonacosane melts at 62.7°C. (Chibnall and Channon, 1929).
n-Octacosane melts at 63°C. (Marker, 1938).
n-Heptacosane melts at 59°C. (Hart and Northrup, 1935).

3.212 mg. dried over P_2O_5 in vacuo gave 10.050 mg. CO_2 and 4.130 mg. H_2O,

and 3.514 mg. similarly dried gave 11.041 mg. CO_2 and 4.522 mg. H_2O.

Found: C, 85.31 and 85.67; H, 14.39 and 14.40%.

Calc. for $C_{29}H_{60}$: C, 85.29; H, 14.71%.

Calc. for $C_{27}H_{56}$: C, 85.26; H, 14.74%.

The/

The second fraction examined was 100 mg. of heavily pigmented material eluted by 30% (v/v) acetone in ether. Most of the coloured material was removed by leaching with 3 ml. of benzene and the residue on crystallization from aqueous ethanol gave 16 mg. of fine rods which melted at 242°C. On admixture with authentic allopregnane-3α,20α-diol it melted at 243°C.

The author has not been able to perform optical rotation measurements as he has not had access to a suitable instrument. Such an instrument has been on order for the Department of Biochemistry for some time and the necessary rotations will be determined as soon as it is delivered.

Discussion.

The isolation of allopregnane-3α,20α-diol from this source was quite simple and it appears in retrospect that the methods employed were perhaps unnecessarily elaborate. Crystallization of the non-ketonic total acetone eluate from the first chromatograph would undoubtedly have given allopregnane-3α,20α-diol though perhaps not in such good yield/

yield. However, the methods employed served a useful purpose in that they involved the handling of many rather small fractions and from this experience several important qualitative observations can be recorded.

Allopregnane-3α,20α-diol and pregnane-3α,20α-diol remained together throughout their preliminary treatment and in particular aluminium oxide chromatography did not cause a separation, although the melting-points of the crude fractions indicated that there may have been relatively more allopregnane-3α,20α-diol in the earlier crystalline fractions. This would be in general agreement with the work of Dobriner and his co-workers (1948) who found that steroids with the allo configuration were eluted from aluminium oxide before their isomers with the normal configuration. However, although many chromatographs have since been performed on diol mixtures, complete separation on aluminium oxide has not been obtained. It has been shown also (Sommerville, Marrian and Kellar, 1948) that allopregnane-3α,20α-diol is precipitated quantitatively with the pregnane-3α,20α-diol in the Astwood-Jones precipitation method used by these authors.

It/

It appears therefore that a binary mixture of the two diols may be obtained fairly readily and that crystallization of this mixture will give one or other in the pure state depending on the relative amounts of each originally present.

Crystallization of the two diols from methanol gives crystalline products which are markedly different. Allopregnane-3α,20α-diol deposits heavy thick glistening rods (for a good photograph see Hartmann and Locher, 1935), while pregnane-3α, 20α-diol gives fine light needles. Further, allo-pregnane-3α,20α-diol appears to go into solution slowly and when in solution allows supersaturation to occur quite readily, while pregnane-3α,20α-diol is both readily soluble in and readily crystallized from methanol. These differences may well be due to the different crystal structure of the two diols and/or to a difference in absolute solubility. Such variations allow allopregnane-3α,20α-diol to be recognized on sight even if a considerable amount of pregnane-3α,20α-diol has crystallized out with it and the difference in rate of solution allows the allo isomer to be purified by leaching with hot methanol.

Summary/

Summary.

1. Allopregnane-3α,20α-diol has been isolated from the products of hydrolysis (HCl) of the acetone mother liquor solids from the preparation of 'sodium pregnanediol glucuronidate' from human pregnancy urine.

2. Cholesterol and a hydrocarbon, probably heptacosane, octacosane or nonacosane, have been isolated from the same source.

Part II.

A METHOD FOR THE DIRECT ISOLATION OF ALLOPREGNANE-3α,20α-DIOL AND ITS USE TO FORM AN ESTIMATE OF THE QUANTITY PRESENT IN HUMAN PREGNANCY URINE.

Previous methods for the isolation of allopregnane-3α,20α-diol involve chemical treatment of a relatively crude diol mixture and subsequent separation of the derivatives of pregnane-3α,20α-diol and allopregnane-3α,20α-diol by differences in their solubility. One such method, which arose from Hartmann and Locher's original work (1935), involves the conversion of the crude diol mixture to the diacetates, removal of pregnane-3α,20α-diol diacetate by crystallization from alcohol and saponification of the mother liquors to get the free allopregnane-3α, 20α-diol. This method has been used by Marker and his co-workers (Marker et al., 1938a) and by Beall (1937). Another method suggested and used by Marker and his co-workers (Marker et al., 1937a) involves the oxidation of the crude diol mixture to the corresponding diones, separation of the disemicarbazones of these diones by crystallization and weighing of the fractions. To apply this second method properly the allo-3β-hydroxy material should be removed, by precipitation with digitonin, before the/

the oxidation step. Each of these methods, therefore, employs several chemical reactions before allopregnane-3α,20α-diol is isolated or estimated, and this of course lessens the significance of the final yield.

It would appear therefore that a method for the isolation of allopregnane-3α,20α-diol which involved no chemical reactions would have considerable advantage if significance was to be attached to the yield. This would be particularly true if only a small amount of the allopregnane-3α,20α-diol was present in the diol mixture.

The work performed in Part I, particularly the qualitative observations recorded in the Discussion, indicated that there were sufficient differences in physical properties between allopregnane-3α,20α-diol and pregnane-3α,20α-diol to give a reasonable hope of evolving a method for the direct isolation of allopregnane-3α,20α-diol. Determination of the solubilities of the two diols in various solvents led to the adoption of a solvent extraction method.

Examination of the solubility figures for allopregnane-3α,20α-diol and pregnane-3α,20α-diol in/

in alcohol showed that if the relative quantities of these two diols in human pregnancy urine were actually those recorded in the literature (Marker et al., 1938b; Pincus and Pearlman, 1943; Fieser and Fieser, 1949b), then crystallization of the total diol mixture from alcohol should give allopregnane-3α,20α-diol as a first crop. Common experience is that pregnane-3α,20α-diol forms the first crop, so the accepted literature values may well be in error. It was decided, therefore, to place this direct method for the isolation of allopregnane-3α,20α-diol on as quantitative a basis as possible and then to use it to re-estimate the quantity in human late pregnancy urine.

Results :

The solubility of pregnane-3α,20α-diol and of allopregnane-3α,20α-diol, both of which had been recrystallized to a constant melting-point, in various solvents was determined by the rapid approximate method recommended by Klyne and Bell (1946). Results are recorded in Table 5.

Table /

Table 5.

Solubility in g./100 ml.

Solvent	Pregnane-3α,20α-diol		Allopregnane-3α-20α-diol	
	At solvent boiling point	At 0°C.	At solvent boiling point	At 0°C.
Methanol	1.8	0.33	0.45	0.07
Ethanol	2.6	0.50	1.0	0.18
Benzene	0.55	0.06 (20°C.)	0.28	0.06 (20°C.)
Acetone		0.03		0.02
Chloroform		0.23 (20°C.)		0.13 (20°C.)

Inspection of these results showed that the two diols had the same solubility in benzene but that there was a five-fold difference in methanol solubility. Considering the close chemical relationship of the two isomers the benzene solubilities require no comment, but the wide difference in their solubilities in alcohols is very surprising. These/

These differences, however, provide a method of separating the two diols.

It appeared that if a diol mixture was thoroughly leached with a volume of benzene insufficient to dissolve all the allopregnane-3α,20α-diol, then the benzene-soluble portion would contain the two diols in 1:1 proportions. Therefore if the benzene solution was separated from the solid material, taken to dryness and the residue crystallized from methanol, about four-fifths of the allopregnane-3α,20α-diol would crystallize out before any mixed crystals were deposited. This was verified experimentally under ideal conditions.

To 700 mg. of pregnane-3α,20α-diol, demonstrated to be free from allopregnane-3α,20α-diol, 13.5 mg. of allopregnane-3α,20α-diol were added and the mixture was homogenized. This mixture was refluxed with benzene and allowed to cool overnight before filtration. The benzene filtrate was taken to dryness and on crystallization from methanol 8.5 mg. of allopregnane-3α,20α-diol were recovered.

Obviously allopregnane-3α,20α-diol could be isolated readily in about 60% yield from such an ideal mixture. Isolation from a urinary source would be much more difficult as the gums and waxes would/

would undoubtedly influence the yield. A control experiment was therefore run on a urine extract to which allopregnane-3α,20α-diol had been added. This experiment was performed as quantitatively as was possible.

A 15-day sample of urine from a post-menopausal woman who had received 1.5 g. of progesterone intramuscularly over the 15 days was obtained. The total neutral ether-soluble portion was obtained from the hydrolysed urine (HCl) and split into two equal portions. To one of these portions 10 mg. of allopregnane-3α,20α-diol was added. The two portions thus obtained were worked up in a strictly parallel manner at the same time.

Each extract was dissolved in benzene and chromatographed through aluminium oxide, only the diol fraction eluted by acetone being collected. The total acetone eluates were refluxed with small volumes of benzene and allowed to crystallize overnight. After filtration the benzene filtrates were chromatographed through small columns of aluminium oxide using mixed solvents of increasing polarity as eluants. All consecutive crystalline fractions were combined and crystallised slowly from methanol. Some allopregnane-3α,20α-diol was isolated/

isolated from each. The mother liquors from these crystallizations which were highly coloured, were taken to dryness and dissolved in a small volume of ethanol at 75°C. to which 4 volumes of water also at 75°C. were added (method of Sommerville, Marrian and Kellar, 1948). This mixture was allowed to stand overnight at 37°C. and the precipitates were then centrifuged down. Recrystallization of the solid from a small volume of methanol gave a further crop of allopregnane-3α,20α-diol from the enriched fraction but none from the other. A second benzene extraction of the remainder of the original material after a similar working up process yielded a small crop of allopregnane-3α,20α-diol from the enriched fraction but none from the other.

In all, 7 mg. of the added 10 mg. of allopregnane-3α,20α-diol were recovered. This 70% recovery indicated that some significance could be attached to the yield of allopregnane-3α,20α-diol obtained by this method from normal human pregnancy urines.

The total neutral ether-soluble portion was obtained from 98 l. of acid hydrolysed (HCl) human late pregnancy urine. The total neutral ether-

soluble/

soluble portion was dissolved in benzene and chromatographed through a column of aluminium oxide retaining only the diol fraction eluted by acetone. This diol fraction was a semicrystalline solid stained deep purple by oily material. It was therefore decided to use the aqueous ethanolic precipitation method (Sommerville, Marrian and Kellar, 1948) at this stage to remove this oily material.

The total solid was therefore dissolved in ethanol at 75°C. and precipitated out by the addition of a four-fold volume of water also at 75°C. and allowed to stand overnight at 37°C. A small amount of filter-aid was added and the mixture was filtered. The precipitate which was still quite highly coloured was refluxed in alcohol with a small amount of 'Norite' charcoal and filtered hot. The filtrate was taken to dryness and then refluxed with dry benzene, allowed to stand overnight and filtered. The benzene filtrate was chromatographed through a column of aluminium oxide retaining only the diol fraction eluted by acetone.

Crystallization of this, and other benzene extractions/

and an adapter carrying a small burette and a reflux condenser. The substance was placed in the flask; the solvent was then run in from the burette while the flask was heated on a sand bath until the solid just dissolved in the boiling solvent. This gave the solubility at the boiling-point. The solution was then cooled to 0°C. and filtered from the solid which had separated. A measured volume of the filtrate was evaporated to dryness, giving the solubility in the cold.

In a typical experiment 9.0 mg. of allopregnane-3α,20α-diol were accurately weighed and placed in the small flask. Dry methanol (1.5 ml.) was added and the mixture was refluxed for 30 min. As crystals were still obviously present a further 0.2 ml. of methanol was added and the refluxing continued. At 10 min. intervals thereafter the hot solution was examined through a magnifying glass and if crystals were still present, a further 0.1 ml. of methanol was added. Solution was complete when a total of 2.0 ml. of methanol had been added. This gave the solubility of allopregnane-3α,20α-diol in boiling methanol as 4.5 mg./ml. or 0.45g./100 ml.

The solution was diluted to 4 ml. with methanol, transferred/

transferred to a narrow tube and allowed to stand at 0°C. for 3 days. A fine sinter glass disc which had been fused on to a fine glass rod and which fitted closely to the sides of the tube was lowered slowly through the solution. A portion (3 ml.) of the clear supernatant liquid was pipetted into a weighed flask, the solvent was evaporated off and the flask reweighed. This 3 ml. of supernatant liquid gave 2.0 mg. of dry residue. Therefore the solubility of allopregnane-3α,20α-diol in methanol at 0°C. is 0.7 mg./ml. or 0.07 g./100 ml.

Each determination was performed in duplicate and the mean figures are recorded in Table 5.

Recovery of added allopregnane-3α,20α-diol:

(a) Ideal conditions.

Pregnane-3α,20α-diol (700 mg., M.P. 236-237°C.) was refluxed with 100 ml. of dry benzene for 1 hr. and allowed to stand at room temperature overnight. The solid was filtered off and retained. The benzene filtrate was evaporated to dryness, dissolved in 7 ml. of methanol and allowed to crystallize in the refrigerator. After 3 days the crop of fine needles (typical of pregnane-3α,20α-diol) was filtered/

filtered off, dried and found to melt at 237°C. A mixture with authentic pregnane-3α,20α-diol (M.P. 236-237°C.) melted at 237-238°C.

To the remainder of the pregnane-3α,20α-diol (c. 670 mg.) 13.5 mg. of allopregnane-3α,20α-diol (M.P. 244-245°C.) were added and the mixture homogenized by complete solution in ethanol followed by evaporation to dryness. This mixture was refluxed for 2 hr. with 100 ml. of dry benzene and allowed to stand at room temperature overnight before filtration. The solid was rebulked with the original material and the benzene filtrate was taken to dryness to give Fraction BS.1. The remaining solid was treated with a further 100 ml. of benzene as above to give Fraction BS.2.

BS.1 was dissolved in 5 ml. of methanol and allowed to stand for 7 days. The thick heavy rod-like crystals which had formed were filtered off, dried,and were found to melt at 242-243°C. They did not depress the melting-point of authentic allopregnane-3α,20α-diol. Yield - 7.0 mg.

BS.2 on similar treatment with 5 ml. of methanol yielded a further 1.5 mg. of crude allopregnane-3α,20α-diol (M.P. 238-241°C.).

The/

The methanol mother liquors from BS.1 and BS.2 were not examined.

8.5 mg. of the added 13.5 mg. of allopregnane-3α,20α-diol were recovered (63%).

(b) Recovery from urine residues.

A post-menopausal woman received 100 mg. of progesterone intramuscularly each day for 15 days. On each day a full 24 hr. specimen of urine was collected under a toluene preservative. The urine was stored in the refrigerator until treated; two or three days' samples were bulked for treatment.

The toluene was removed from each urine sample by means of a large separating funnel. Urine volume was noted and it was transferred to a large conical flask (batches of from 3-4 l. were used). The urine was brought to the boil, 10%(v/v) of concentrated HCl was added and the mixture boiled for 10 min. The hydrolysate was cooled rapidly and extracted 3 x 1/3 volume of peroxide-free ether. The combined ether extracts were washed 3 x 1/3 volume of N.NaOH and then 3 x 1/3 volume of distilled water. The ether was distilled off leaving the neutral ether-soluble/

soluble portion. The various individual portions were bulked to give a total neutral ether-soluble fraction, a red gum which weighed 1.243 g.

The total neutral ether-soluble fraction was completely dissolved in 100 ml. of ethanol. 50 ml. of the ethanolic solution were placed in flask I and the remainder in flask G. Allopregnane-3α,20α-diol (10 mg.) was added to flask G and the contents of both flasks were evaporated to dryness and received the following treatment at the same time.

The material was dissolved in 100 ml. of dry benzene and absorbed from it on to a column of 20 g. of slurry-packed Grade II Al_2O_3 (Brockmann, 1941). The column was eluted with dry benzene (1000 ml.) and dry ether (1000 ml.) until no more material was eluted. The crude diol fraction was then eluted by 350 ml. of 15% (v/v) methanol in acetone; no solid was present in the last 50 ml. of this fraction.

The crude diol fraction was refluxed for 2 hr. with 25 ml. of dry benzene and allowed to stand overnight at room temperature before filtering. The solid was returned to the original bulk of material and the benzene filtrate was poured through a 5 g. column of slurry-packed Grade II Al_2O_3 (Brockmann, 1941). The chromatograph was developed with 200 ml. dry/

dry benzene, 100 ml. of 50% (v/v) ether in benzene and by 100 ml. dry ether (no crystalline material was eluted by the 100 ml. of ether). The diol mixture was then eluted by 25 ml. portions of 5% (v/v) methanol in ether, the first three such fractions contained crystalline material and were bulked as BS.1.

The remainder of the crude diol fraction was refluxed with a further 25 ml. of dry benzene, allowed to stand overnight at room temperature and filtered. The solid was returned to the original bulk and the filtrate was taken to dryness as BS.2. A BS.3 fraction was obtained from a 25 ml. benzene extraction of the remainder of the diol fraction in the same way.

The material from flask I (no added allopregnane-3α,20α-diol) gave 390 mg. of a brown-red crystalline mass as a crude diol fraction. BS.1, a purple oily solid, was dissolved in 3 ml. of methanol and allowed to evaporate down to about 0.5 ml. over 3 days. The highly coloured mother liquors were decanted off and the solid on recrystallization from 2 ml. of methanol (3 days) gave 1.0 mg. of thick rods which melted at 238-243°C. and which did not/

not significantly depress the melting point of authentic allopregnane-3α,20α-diol. The methanol mother liquor solids from both these treatments were dissolved in 2 ml. of ethanol at 75°C. and precipitated out by 8 ml. of water also at 75°C. After standing overnight at 37°C. the solid was spun down on the centrifuge, dried and dissolved in 1 ml. methanol. Even after one week in the refrigerator no crystalline material formed, so this material was discarded.

BS.2 was taken up in 5 ml. of methanol and allowed to evaporate slowly to 2 ml. during 3 days. The crystalline solid was filtered off, dried and found to melt at 234-237°C. and did not depress the melting-point of authentic pregnane-3α,20α-diol. The mother liquors were discarded.

BS.3 also yielded only pregnane-3α,20α-diol.

The material in flask G (10 mg. of allo-pregnane-3α,20α-diol added) gave 330 mg. of a brown-red crystalline mass as a crude diol fraction. BS.1, a purple oily solid, was dissolved in 3 ml. of methanol and allowed to evaporate down to 0.5 ml. (3 days) when the highly coloured mother liquors were/

were decanted off. The solid on recrystallization from 2 ml. of methanol gave 3.6 mg. of thick rods, still a little purple in colour, which melted at 239-242°C. and when mixed with authentic allopregnane-3α,20α-diol (M.P. 244-245°C.) melted at 240-242°C. The mother liquor solids from both these methanol treatments were dissolved in 2 ml. of ethanol at 75°C. and precipitated out by the addition of 8 ml. of distilled water also at 75°C. After standing overnight at 37°C. the solid was spun down in the centrifuge, dried and dissolved in 1 ml. methanol. After 4 days in the refrigerator 3.8 mg. of thick glistening rods were isolated, found to melt at 240-244°C. and did not depress the melting-point of authentic allopregnane-3α,20α-diol.

BS.2 was taken up in 5 ml. of methanol and allowed to evaporate down to 2 ml. (3 days) when the mother liquors were decanted off. The solid when dried was a mixture of sandy crystals and fine rods and melted at 224-233°C. This solid when allowed to crystallize slowly from methanol (2 ml.) gave a few rod-like crystals (0.6 mg.) which melted at 241-243°C. and did not depress the melting-point of/

of authentic allopregnane-3α,20α-diol. The mother liquors were not further examined.

Treatment of BS.3 gave only pregnane-3α,20α-diol.

Total yield of allopregnane-3α,20α-diol from the material in flask I (no added allopregnane-3α, 20α-diol) was 1.2 mg.

Total yield of allopregnane-3α,20α-diol from material in flask G (10 mg. of allopregnane-3α,20α-diol added) was 8.0 mg.

Therefore 7 mg. of the added 10 mg. had been recovered (70%).

Quantity of allopregnane-3α,20α-diol in human late pregnancy urine.

The total neutral ether-soluble portion was obtained from 98 l. of acid hydrolysed (HCl) pooled human late pregnancy urine in the manner previously described (p. 49). This yielded 10.5 g. of deep brown-red gum.

The total portion was dissolved in 500 ml. of dry benzene and poured through a 300 g. column of slurry-packed Grade II Al_2O_3 (Brockmann, 1941). The column was developed with benzene and then with ether until the deep purple band which normally marks the diol band started to move slowly down the column.

The/

The crude diol fraction was then obtained by elution with acetone until 100 ml. of the eluate gave no residue when evaporated to dryness. This crude diol fraction was a deep purple crystalline mass weighing 3.09 g.

This whole fraction was dissolved in 1000 ml. of ethanol at 75°C., precipitated by the addition of 4000 ml. of distilled water also at 75°C. and stood overnight in the incubator at 37°C. A small amount of 'Supercel' filter-aid (6 g.) was added, the mixture vigorously shaken and allowed to stand at 37°C. for a further 2 hr. before filtration through a large sinter glass funnel. The filtrate was discarded and the diols were washed through the sinter glass funnel by boiling ethanol. The resulting solid, after removal of the ethanol by evaporation, was still highly coloured. This material was dissolved in 250 ml. of ethanol, 3 g. of 'Norite' charcoal were added, and the mixture was refluxed for half an hour before filtration, while still hot, through a fluted filter paper. The filtrate on evaporation to dryness gave 2.55 g. of brown crystalline solid.

This material was refluxed for 2 hr. with 200 ml. of dry benzene and allowed to stand overnight before/

before filtering. The solid was returned to the original bulk and the filtrate was poured through a 1 g. column of slurry-packed Grade II Al_2O_3 (Brockmann, 1941) and the diol fraction obtained by elution as previously described (pp. 50-51) to give BS.1.

BS.1 was dissolved in 7 ml. of methanol and allowed to evaporate slowly (10 days) down to a volume of about 1.5 ml. when the highly coloured mother liquors were decanted off. These mother liquors were retained as A while the solid on slow crystallization from 5 ml. of methanol yielded 21 mg. of thick rods which melted at 235-242°C. and which did not significantly depress the melting-point of authentic allopregnane-3α,20α-diol. The mother liquors from this second crystallization were added to the main bulk of the diol fraction which was then refluxed with 100 ml. of dry benzene for 1 hr., left overnight and filtered. The solid was rebulked as the diol fraction and the filtrate when taken to dryness gave fraction BS.2.

BS.2 was dissolved in 7 ml. methanol and allowed to evaporate slowly (3 days)down to a volume of 2 ml. when the coloured mother liquors were decanted off. These/

These mother liquors were retained as B while the solid on recrystallization from 5 ml. methanol gave 6.5 mg. of thick rods which melted at 237-242°C. and which did not depress the melting-point of authentic allopregnane-3α,20α-diol. The methanol mother liquors from this second crystallization were returned to the original bulk of diol material from which a BS.3 fraction was obtained by treatment with a further 100 ml. of benzene.

BS.3 on direct slow crystallization from methanol gave a crop of fine needles which melted at 235-236°C. and which did not depress the melting-point of authentic pregnane-3α,20α-diol. This whole fraction was therefore returned to the bulk of the diol mixture.

The mother liquor materials A and B were bulked, dissolved in 4 ml. of methanol and allowed to stand at constant volume for 10 days. This yielded 18 mg. of thick light-brown rods which melted at 237-242°C. and which did not depress the melting-point of authentic allopregnane-3α,20α-diol. The mother liquors were returned to the original diol mixture.

The/

The original diol mixture, which had now yielded 45.5 mg. of allopregnane-3α,20α-diol but which still contained all the pregnane-3α,20α-diol initially present, was dissolved in 250 ml. of ethanol at 75°C. and precipitated out by the addition of 1000 ml. of distilled water also at 75°C. This mixture stood overnight in the incubator at 37°C. and was filtered through a sinter glass funnel. The filtrate was discarded. The solid was washed through the funnel by a jet of boiling ethanol and on evaporation of the ethanol 1.705 g. of light brown crystalline material was left.

This diol fraction was refluxed with 100 ml. dry benzene and treated as previously described to give fraction BS.4. BS.4 on slow crystallization from 4 ml. of methanol yielded 7 mg. of thick rods which melted at 239-241°C. and which did not depress the melting-point of authentic allopregnane-3α,20α-diol. On reduction of the mother liquor volume to 2 ml. crystalline pregnane-3α,20α-diol was obtained so the complete mother liquor material was rebulked with the diol fraction. This diol fraction was treated with 50 ml. benzene as previously described to give BS.5. Slow crystallization of BS.5 from methanol gave only pregnane-3α,20α-diol and so was rebulked/

rebulked with the diol fraction.

The remaining bulked diol fraction (1.70 g.), although slightly brown in colour, melted at 235-237°C. and did not depress the melting-point of authentic pregnane-3α,20α-diol.

Yields: A total of 52.5 mg. of allopregnane-3α,20α-diol from 98 l. of human late pregnancy urine or 0.54 mg./l.

A total of 1.70 g. of pregnane-3α,20α-diol from 98 l. of human late pregnancy urine or 17.4 mg./l. In view of the fact that this material was not absolutely pure the figure of 17 mg./l. will be used.

The ratio Pregnane-3α,20α-diol isolated: allopregnane-3α,20α-diol isolated is therefore 17:0.54 or approximately 30:1.

Discussion /

Discussion.

The solubility figures determined for pregnane-3α,20α-diol and allopregnane-3α,20α-diol in several solvents were, although only approximate, sufficient to allow the method to be devised. The surprisingly wide difference in the solubility of the two isomers in alcohol was an unexpected but fortunate factor and formed the basis of the method. The work served to re-emphasize the slowness of the crystallization of allopregnane-3α,20α-diol and also indicated that oils and waxes tended to hold it in supersaturated solution.

The figures obtained for the recovery of added allopregnane-3α,20α-diol, both under the ideal conditions where maximum recovery was not seriously attempted and from urinary extracts where the 70% recovery represented a serious attempt to attain the maximum, indicated that the yield obtained from human pregnancy urine could be accepted as a measure of the amount actually present. It is felt, moreover, that as the allopregnane-3α,20α-diol recovery from the control experiment resulted from but two benzene treatments the isolation from the urine may well have been more quantitative. The greater number of benzene/

benzene extractions, with the return of the methanolic mother liquors before each benzene treatment, would probably result in the same absolute loss which, because of the larger amount of allopregnane-3α,20α-diol present, would be relatively less significant. It is of course true that the actual amount of allopregnane-3α,20α-diol isolated (0.54 mg./l.) from the human late pregnancy urine must represent the minimum quantity present. Several isolations of allopregnane-3α,20α-diol from human pregnancy urine have now been carried out by the method employed here, in order to obtain a stock of material, and yields approaching 0.5 mg./l. have been obtained several times; a yield in excess of 0.54 mg./l. has not been obtained. This provides additional support for the belief that the yield obtained in this instance approached the maximum. It is therefore suggested that the concentration of allopregnane-3α,20α-diol in human late pregnancy urine is 0.6-0.8 mg./l.

This estimate is strikingly different to that recorded by Marker (Fieser and Fieser, 1949[b]) and suggests that whatever factors caused him to record such a high figure for the allopregnane-3α,20α-diol in/

in mares' pregnancy urine in 1937 (Marker et al., 1937a), (and he himself has shown that such factors did exist although he does not record what they were (Marker et al., 1939)), must have operated in the isolation from human pregnancy urine which he records (Fieser and Fieser, 1949b).

The ratio (30:1) of pregnane-3α,20α-diol isolated: allopregnane-3α,20α-diol isolated cannot be regarded as a precise relationship as minor losses during the isolation, particularly of the allopregnane-3α,20α-diol, which would not significantly affect the final absolute figures, would cause marked differences in the ratio.

It may be noted that the figures here obtained for the quantity of pregnane-3α,20α-diol in human pregnancy urine (17 mg./l.) is higher than the figure recorded by Marker (13.6 mg./l. Fieser and Fieser, 1949b). However, in view of the rapid increase in the urinary excretion of pregnane-3α,20α-diol during the later stages of pregnancy this may only indicate that the urine used by the author came from patients closer to term than that used by Marker.

Summary/

Summary.

1. A solvent extraction method has been devised for the isolation of allopregnane-3α,20α-diol from crude diol mixtures.

2. Allopregnane-3α,20α-diol has been isolated from human late pregnancy urine in a yield of 0.54 mg./l. This yield is believed to be close to the maximum possible.

3. Pregnane-3α,20α-diol and allopregnane-3α,20α-diol have been isolated from human late pregnancy urine in the ratio 30:1.

4. It is suggested that the true concentration of allopregnane-3α,20α-diol in human late pregnancy urine is 0.6-0.8 mg./l.

Part III.

THE REMOVAL OF ALLOPREGNANE-3α,20α-DIOL FROM 'SODIUM PREGNANEDIOL GLUCURONIDATE'.

The isolation of allopregnane-3α,20α-diol reported in Part I was accomplished easily from the neutral free steroid fraction obtained after the hydrolysis of the material retained in the 95% acetone mother liquors, which occur in the preparation of 'sodium pregnanediol glucuronidate' by the conventional method. The isolation was so simple that a considerable alteration of the relative proportions of allopregnane-3α,20α-diol and pregnane-3α,20α-diol must have taken place during the working up of the urine. This implied that allopregnane-3α,20α-diol, or its complex, could be removed from 'sodium pregnanediol glucuronidate' by acetone precipitation. It appeared necessary to investigate just how efficient this removal was.

Results.

A batch of 'sodium pregnanediol glucuronidate' was isolated from 78 l. of human late pregnancy urine by the conventional method of butanol extraction followed by precipitation of the butanol soluble material/

material, after the removal of acids and phenols, from 50% (v/v) acetone solution by acetone. After a second such acetone precipitation 4.5 g. of almost white 'sodium pregnanediol glucuronidate' was obtained. This material melted at 260°C. (long stem uncorrected) with decomposition.

The 'sodium pregnanediol glucuronidate' so obtained was dissolved in water, hydrolysed with HCl and the neutral ether-soluble material isolated. The neutral ether-soluble material was separated into a ketonic and a non-ketonic fraction by a Girard separation (Girard and Sandulesco, 1936). The non-ketonic material was further purified by precipitation from ethanolic solution by water following the method of Astwood and Jones (1941). The non-ketonic material was refluxed with a small quantity of benzene and allowed to crystallize from it. The benzene-soluble portion, on taking to dryness and crystallization of the solids from methanol, gave a good yield of allopregnane-3α,20α-diol.

Since the foregoing experiment showed that 'sodium pregnanediol glucuronidate' prepared and purified in the normal manner still contains allopregnane-3α,20α-diol, or its complex, an experiment was/

was carried out to see if the allopregnane-3α,20α-diol could be removed by more rigorous methods of purification.

A large mixed batch of various samples of 'sodium pregnanediol glucuronidate' was obtained. The history of several of the individual batches was obscure, but all had been submitted to at least two precipitations from aqueous acetone solution by acetone. The entire batch was submitted to three more precipitations from aqueous acetone solution by acetone. The resultant material was a white crystalline solid which melted at 263-265°C. with decomposition (long stem, uncorrected). A portion of this material was retained as Fraction A.

The remainder of this pure 'sodium pregnanediol glucuronidate' was subjected to two crystallizations from 90% (v/v)ethanol to give Fraction B which melted at 266-268°C. with decomposition (long stem uncorrected). The 90% (v/v) ethanolic mother liquors from B were examined as Fraction BM.

Fractions A, B and BM were dissolved in water, hydrolysed with HCl and the neutral ether-soluble material obtained from each. Each neutral ether-soluble portion was separated into a ketonic and a non-ketonic/

non-ketonic fraction using trimethylammoniumaceto-hydrazide chloride (Girard's Reagent 'T'). The ketonic fractions were not examined beyond the obtaining of a stock of pregnane-3α-ol-20-one by direct crystallization. The portions of the non-ketonic fractions soluble in small volumes of benzene were taken to dryness and examined for the presence of allopregnane-3α,20α-diol by crystallization from methanol. No allopregnane-3α,20α-diol was found in Fraction A, B or BM.

However, from each fraction methanol treatment of the solid from the first benzene extraction gave a material, extremely insoluble in methanol, which melted, in the case of Fractions A and B at 290°C. with much sublimation, and in the case of Fraction BM at 270°C. with much sublimation. Although mixed melting-points were not very reliable when applied to these materials, because of the rapid sublimation each one underwent before melting, they indicated that the materials from A and B were the same; they were therefore bulked as Fraction KA (7.0 mg.). The melting-point data on the material from Fraction BM did not justify its inclusion in KA/

KA: so as it weighed only 1.1 mg. it was not further examined.

Fraction KA sublimed evenly under high vacuum and gave a negative Zimmermann reaction which may, however, have been negative because of the extreme insolubility of the material in ethanol. It gave analytical figures which agreed fairly well with those required for a formula $C_{21}H_{34}O_3$. Evidence from the infra-red absorption of acetylated KA indicates that it is a saturated steroid which may have a tertiary and an 11β-hydroxy group, while the possibility of a 17-keto group was not completely eliminated. KA has still to be examined further.

Experimental.

Preparation of 'sodium pregnanediol glucuronidate'

Batches of human late pregnancy urine, preserved with butanol, were extracted 3 x 1/3 vol. of butanol as soon as possible after collection. The butanol extracts were evaporated under reduced pressure to dryness. The residue was dissolved in 1/5 of the original urine volume of 0.5 N.NaOH and extracted 3 x 1/3 vol. of butanol. The combined butanol extract was washed 3 x 1/12 vol. of distilled H_2O/

H_2O, 12 hr. was allowed for each wash and subsequent separation. The butanol was evaporated to dryness under reduced pressure. The material obtained in this way was dispersed with warming in a small volume of 50% (v/v) aqueous acetone and the solution filtered hot. Crude 'sodium pregnanediol glucuronidate' was precipitated from the filtrate by the addition of 10 vol. of dry acetone. After standing overnight the precipitate was filtered by suction and washed with dry acetone.

Batches equivalent to 78 l. of pregnancy urine were obtained, bulked in 100 ml. of 50% (v/v) aqueous acetone and precipitated by the addition of 1 l. of dry acetone.

Yield: 4.50 g. of off-white crystals which melted at 260°C. with decomposition (long stem, uncorrected).

Preparation of the neutral ether-soluble portion.

The 'sodium pregnanediol glucuronidate' was dissolved in distilled water to give a solution of concentration approximately 0.3 g./l. This solution was hydrolysed in 3 l. batches.

The aqueous solution (3 l.) was heated to the boiling point, 300 ml. of concentrated HCl were added/

added and the boiling continued for 10 min. This hydrolysate was cooled rapidly and extracted thrice with 1000 ml. lots of peroxide-free ether. After washing twice with 400 ml. lots of 0.5 N.NaOH and three times with 400 ml. lots of distilled water, the combined ether extract was evaporated to dryness, leaving the neutral ether-soluble portion.

The 4.50 g. of 'sodium pregnanediol glucuronidate' previously prepared yielded 2.265 g. of a white solid.

Girard Separation.

The neutral ether-soluble portion (2.265 g.) was dissolved in a mixture of 50 ml. of ethanol and 5 ml. of glacial acetic acid. Trimethylammonium acetohydrazide chloride (8 g.) was added and the mixture refluxed on a boiling water bath for 60 min. and then cooled rapidly. A solution of 3.15 g. NaOH (sufficient to neutralize 9/10 of the acetic acid) in 200 ml. of ice-cold water was then added and the mixture transferred to a separating funnel containing a further 800 ml. of ice-cold water. The mixture was extracted four times with 500 ml. portions of ether. The combined ether extracts were/

were washed once with 300 ml. of 0.5 M. $NaHCO_3$ and three times with 300 ml. portions of distilled water and evaporated to dryness. The non-ketonic fraction obtained thus weighed 1.620 g.

The aqueous phase was acidified with 30 ml. concentrated H_2SO_4 and allowed to stand 3 hr. at room temperature. The mixture was extracted three times with 500 ml. portions of ether. The combined ether extracts were washed once with 200 ml. of 0.5 M. $NaHCO_3$ and three times with 200 ml. portions of distilled water and distilled to dryness. The ketonic fraction thus obtained was crystallized from n-hexane and the pregnane-3α-ol-20-one obtained was stored.

The non-ketonic fraction (1.620 g.) was dissolved in 1 l. of ethanol at 75°C., 4 l. of distilled water also at 75°C. were added slowly with violent shaking and the mixture stood overnight in an incubator at 37°C. The flocculent precipitate was filtered off into a large sinter glass funnel by suction and was dried in the funnel after washing with 20% (v/v) aqueous ethanol. The material was dissolved from the funnel by boiling ethanol. The non-ketonic fraction now weighed 1.341 g.

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The non-ketonic fraction was refluxed for 1 hr. with 100 ml. dry benzene and allowed to stand at room temperature overnight. The solid was filtered off by suction and returned to the original flask. The benzene filtrate was taken to dryness. The benzene soluble fraction obtained thus weighed 250 mg.

This benzene soluble fraction readily dissolved in 15 ml. of warm methanol, but on cooling there was an immediate precipitate of waxy crystals. This material was probably the hydrocarbon which has been met previously (Part I) in urinary extracts, so the methanol was evaporated off and the solid material was leached with 5 ml. of n-hexane and filtered. The filtrate was not examined. The hexane-insoluble material which weighed 73.5 mg. was dissolved in 10 ml. of hot methanol and allowed to crystallize in the refrigerator over several days. The crystalline crop was filtered off, using suction and when dry weighed 4.0 mg. The material had crystallized in rod-like crystals which melted at 237-242°C. A mixture with authentic allopregnane-3α,20α-diol (M.P. 243°C.) melted at 240-243°C. and

a/

a mixture with authentic pregnane-3α,20α-diol (M.P. 236°C.) melted at 215-217°C.

The remainder of the non-ketonic fraction was refluxed with 200 ml. of dry benzene, allowed to stand overnight and filtered. The benzene filtrate was taken to dryness and left a residue which weighed 96 mg. This residue on crystallization from methanol gave a further 4 mg. of good allopregnane-3α,20α-diol.

Purification of a large batch of 'sodium pregnanediol glucuronidate'.

Several specimens of 'sodium pregnanediol glucuronidate' at various stages of purity were bulked to give the starting material. Each individual specimen was known to have been precipitated from aqueous acetone solution by acetone at least twice.

The entire batch (19.4 g.) was dissolved in 600 ml. of hot 50% (v/v) aqueous acetone and filtered, while still hot, through a glass wool pad into a 10 l. flask. The glass wool was finally washed with 100 ml. of hot 50% (v/v) aqueous acetone. Dry acetone (7 l.) was added slowly and with shaking to the filtrate and the mixture was allowed to stand overnight/

overnight. The fairly clear supernatant liquid was siphoned off through a sintered glass funnel and the sludge finally transferred to it using dry acetone as a wash liquid. The solid was dried and returned to the original 10 l. flask where two further precipitations from aqueous acetone solution by acetone were performed, using 700 ml. of 50% (v/v) aqueous acetone for solution and 7 l. of dry acetone for precipitation in each case.

Yield was 13.49 g. of white crystalline material melting at 263-265°C. with decomposition and evolution of gas (long stem, uncorrected).

4.84 g. of this material was set aside as Fraction A.

The remainder of the material (8.65 g.) was dissolved in the minimum volume (1000 ml.) of 90% (v/v) aqueous ethanol at its boiling point and allowed to stand at room temperature for three days. The mixture was then stood at 0°C. for two hours and the crystalline material was filtered off by suction. The total solid was redissolved in 800 ml. of 90% (v/v) aqueous ethanol at its boiling point and allowed to stand overnight at 0°C. The resulting crystalline material, when filtered off and dried, constituted Fraction B (2.75 g.) which melted at 266-268°C. with decomposition. and evolution of

gas/

gas (long stem, uncorrected).

The 90%(v/v) aqueous ethanolic mother liquors from both crystallizations were bulked and evaporated to dryness to give a white crystalline solid which weighed 5.90 g. and was dealt with as Fraction BM.

Treatment of Fraction A.

The purified 'sodium pregnanediol glucuronidate' constituting Fraction A (4.84 g.) was acid hydrolysed by the general method previously described and yielded a total neutral ether-soluble fraction which weighed 2.553 g. This 2.553 g. of material was divided into a ketonic and non-ketonic fraction by means of the Girard separation previously described, with appropriate alterations in the quantities of reagents to compensate for the different weight of starting material. The non-ketonic fraction weighed 2.053 g.

This non-ketonic fraction was refluxed with 100 ml. of dry benzene for 2 hr. and allowed to stand overnight. The solid was filtered off by suction and returned to the original flask. The filtrate was run through a column of 2 g. of Grade II (Brockmann/

(Brockmann, 1941) Al_2O_3, the column was well eluted with dry ether and the total acetone fraction was taken to dryness and retained as BS.1 (102 mg.). (The chromatograph was inserted to remove any hydrocarbon-like substances and the small amount of pregnane-3α-ol-20-one which occasionally do persist even after such rigorous treatment). A second crystallization of the remainder of the non-ketonic material from 100 ml. of dry benzene by the method given above was performed. The benzene filtrate on evaporation to dryness gave Fraction BS.2 (88 mg.).

BS.1, a lightly coloured rather oily solid, was dissolved in 8 ml. of methanol and allowed to stand at room temperature for several days. A deposit of rather sandy looking crystals appeared which would not redissolve in the methanol even though the volume was greatly increased and the mixture warmed. They were filtered off by suction and dried. Yield was 4.0 mg. of white needles which on heating in the melting-point apparatus softened a little at 230°C., started to sublime away quite rapidly at 270°C. and melted, with decomposition, at 287-290°C. The methanol mother liquors from this isolation when reduced in volume gave a good crop of fine needles which melted

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at 233-236°C. and which did not depress the melting-point of good pregnane-3α,20α-diol.

BS.2 on crystallization from a small volume of methanol also provided a crop of good pregnane-3α,20α-diol,and on reducing the volume of the mother liquors a second crop of the same material was obtained.

In all the methanol crystallizations ample time was allowed for any allopregnane-3α,20α-diol, which appears to come out of solution rather slowly, to form crystals.

No allopregnane-3α-20α-diol was isolated from the hydrolysis products of Fraction A.

Treatment of Fraction B.

The highly purified 'sodium pregnanediol glucuronidate' which constituted Fraction B (2.75 g.) gave, on acid hydrolysis by the general method previously described, a total neutral ether-soluble portion which weighed 1.51 g. This gave after a Girard separation, by the method previously described with the modifications necessary for treating a smaller sample, a non-ketonic fraction which weighed 1.09 g.

The/

The whole non-ketonic fraction was refluxed with 50 ml. dry benzene for 1 hr. and allowed to stand overnight and a fraction BS.1 (55 mg.) was obtained as previously described. Refluxing of the remainder of the non-ketonic fraction with a further 50 ml. of dry benzene and treatment as previously described gave fraction BS.2 (34 mg.).

BS.1 was dissolved in 7 ml. of methanol and allowed to stand at room temperature for several days. A sandy crystalline material was deposited which was apparently insoluble in even a large volume of methanol. This material was filtered off and dried. Yield was 3.0 mg. of a sandy type of crystal, slightly off-white in colour, which started to sublime away rapidly at 275°C. and melted with decomposition at 290°C. The methanol mother liquors on reduction of their volume gave a crop of fine white needles which melted at 235-236°C. and which did not depress the melting-point of pure pregnane-3α,20α-diol.

BS.2 on crystallization from methanol also yielded a good crop of pregnane-3α,20α-diol.

No allopregnane-3α,20α-diol was isolated from the hydrolysis products of Fraction B.

Treatment/

Treatment of Fraction BM.

This ethanolic mother liquor material (5.90 g.) when treated as described above for Fractions A and B, gave a neutral ether-soluble portion weighing 4.0 g. and a non-ketonic fraction weighing 2.42 g.

Fractions BS.1 (94 mg.) and BS.2 (62 mg.) were obtained in the manner described previously using 100 ml. of benzene to obtain each.

BS.1 on standing in 8 ml. of methanol deposited fine white crystals which were almost insoluble in quite a large volume of methanol. They were filtered off to give a yield of 1.1 mg. of fine white needles which started to sublime away at 250°C. and appeared to melt at 270-272°C. with decomposition. (This melting-point was difficult to observe as the sublimation became very rapid above 260°C). The methanol mother liquors gave a good crop of pregnane-3α,20α-diol when their volume was reduced.

BS.2 provided only pregnane-3α,20α-diol on crystallization from methanol.

No allopregnane-3α,20α-diol could be isolated by treatment of Fraction BM.

Treatment/

Treatment of the fractions with high melting-points.

A mixture of the high melting-point fractions from A and B apparently still melted in the region about 290°C. The rate of sublimation below the melting-point was so great, and consequently the rate of heating employed was so high, that no definite conclusion could be drawn from this observation. It was felt, however, on general grounds that they must be the same so they were bulked to give 7 mg. of material designated KA.

The high melting-point material from Fraction BM (1.1 mg.), which had a lower melting-point (270°C.) than either of the other two, was not examined as it was not felt that it could be added to KA and there was insufficient material to allow it to be examined as an individual.

KA when heated slowly in high vacuum yielded 6.3 mg. of fine white material which sublimed evenly over the range 140°C. to 160°C. at 3×10^{-3} mm. of mercury. This material melted, on rapid heating, at 294°C. with decomposition.

KA (0.05 mg.) in 4 drops of aldehyde-free ethanol was mixed with 2 drops of 2.5 N. ethanolic KOH /

KOH and 2 drops of 2% ethanolic m-dinitrobenzene and allowed to stand for one hour. The pale brown colour which developed was certainly no deeper than that given by a reagent blank. As KA was extremely insoluble in ethanol some solid particles were present in the mixture and they remained white. This doubtfully netative Zimmermann test when taken in conjunction with the fact that the material was isolated from non-ketonic fractions from Girard separations enables the conclusion to be drawn that KA is non-ketonic.

1.233 mg. gave 3.407 mg. CO_2 and 1.188 mg. H_2O.

Found: C, 75.4; H, 10.7%.

Calc. for $C_{21}H_{34}O_3$: C, 75.4; H, 10.2%.
" $C_{21}H_{36}O_3$: C, 75.0; H, 10.7%.

1.0 mg. of KA was dispatched to Dr Konrad Dobriner who obtained the infra-red absorption of acetylated KA and provided the following report: "Concerning the infra-red absorption of the sample you sent, we had to acetylate it because it was insoluble in carbon disulphide. The acetylation was done in pyridine and acetic anhydride in the usual way.

Substance KA gives an absorption in the fingerprint region which we have seen on several occasions/

"occasions and have given it code number 513E20. The material is not very soluble in carbon disulphide. It shows a weak hydroxyl absorption after acetylation suggesting that a tertiary hydroxyl and an 11β-hydroxyl group are present, both of which do not acetylate under the conditions described. This statement should be taken with some reservation because it is only true if acetylation was complete. There is a carbonyl absorption at 1743 cm^{-1} and a strong absorption at 1236-1239. This combination confirms the presence of one or more acetylated groups, but the possibility of the presence of a 17-keto group in the molecule cannot be disregarded. There is no evidence of unsaturation. I would suggest that you send me a small amount, a milligram or so, of well acetylated material for checking purposes. I have discussed the absorption of this compound with Dr Norman Jones."

This material has still to be examined further.

Discussion.

The isolation of allopregnane-3α,20α-diol from fairly crude, but not from pure 'sodium pregnanediol glucuronidate' indicates that the acetone precipitation method of purification does gradually remove the allopregnane-3α,20α-diol or its complex. It was not felt that a determination of the minimum number of acetone treatments required would have any great significance as the efficiency of the removal will depend on the volume of acetone used and probably on the amount of gummy material extracted from the urine. Each of these factors will show a variation from worker to worker and from urine to urine. It is merely reported, therefore, that two acetone treatments of crude 'sodium pregnanediol glucuronidate' are insufficient to remove allopregnane-3α,20α-diol but five such treatments are ample.

The isolation of a trioxygenated C_{21} steroid with such a high melting-point from the rigorously purified 'sodium pregnanediol glucuronidate' is interesting. As the urines used were all obtained from normal human pregnancy cases approaching term, this steroid must, in the first instance, be regarded as a normal constituent of such urines. However,

the/

the possibility of it being an artefact due to the age or treatment of the starting material must be borne in mind. The quantity isolated (8 mg.), which probably approached the maximum because of the very low solubility of the material, indicates that it must be present in a very low concentration in the urine as the 19.4 g. of starting material must have arisen from a minimum of some 400 l.; the concentration must therefore be of the order of 0.02 mg./l.

The lack of information on the structure of this material is disappointing as it was felt that a melting-point so much higher than that of the more common steroids probably indicated some marked difference in structure which would be readily detected by physical methods. D-ring enlargements in C_{21} steroids having a 17-tertiary hydroxyl group have been reported by several workers (Hirschmann and Hirschmann, 1947), and as such D-homo steroids generally have higher melting-points than their parent steroid, this was felt to be quite a strong possibility. However, the report from Dr Konrad Dobriner does not provide any support for this view.

If/

If further supplies of KA can be obtained an attempt to elucidate its structure will be made.

Summary.

1. The precipitation and reprecipitation of 'sodium pregnanediol glucuronidate' from aqueous acetone solution by acetone does remove allopregnane-3α,20α-diol, or its complex. Two such precipitations are insufficient but five are ample for complete removal.

2. A steroid having the probable general formula $C_{21}H_{34}O_3$ or $C_{21}H_{36}O_3$ has been isolated from the hydrolysis products from rigorously purified 'sodium pregnanediol glucuronidate'. This steroid must, in the first instance, be regarded as a normal trace constituent of human pregnancy urine.

Part IV.

PROGESTERONE AS A METABOLIC PRECURSOR OF ALLOPREGNANE-3α,20α-DIOL.

The administration of progesterone (Venning, 1937), cholesterol (Bloch, 1945) and desoxycorticosterone (Cuyler et al., 1940) to human subjects has been shown to result in a significant rise in the urinary excretion of pregnane-3α,20α-diol. No claim has been made that any of these steroids is a precursor of allopregnane-3α,20α-diol, nor has any work been reported which has attempted to find such a precursor. That no such work has been done is undoubtedly due to the difficulty of isolating allopregnane-3α,20α-diol by the conventional methods and relating the yields obtained to the amount originally present in the urine.

It would seem reasonable in view of the close chemical relationship between pregnane-3α,20αdiol and allopregnane-3α,20α-diol to suggest that they could arise from the same metabolic source or sources. That one such source could be progesterone is highly probable although the fact that the allo configuration is common to most of the saturated steroids so/

so far isolated and related to the adrenal cortex suggests that a separate corticoid precursor for allopregnane-3α,20α-diol is also a possibility.

It was decided to use the direct method of isolating allopregnane-3α,20α-diol previously evolved (Part II) to determine whether progesterone is a precursor of allopregnane-3α,20α-diol. The method was applied in as quantitative a manner as possible as it was felt that the amount of allopregnane-3α,20α-diol produced, in relation to both the progesterone dose and to the amount of pregnane-3α,20α-diol produced, would hold some interest.

Results

Three patients having little or no endogenous pregnane-3α,20α-diol and therefore presumably no endogenous allopregnane-3α,20α-diol were chosen.

Patient A: a normal postmenopausal woman.
Patient B: a male with chronic rheumatoid arthritis.
Patient C: a postmenopausal woman with rheumatoid arthritis.

The restricted movement of each patient due to age and/or arthritis enabled full 24 hr. urine specimens to be collected daily.

A daily dose of progesterone was administered intramuscularly to each patient for a period of about

14/

14 days. Full 24 hr. specimens of urine were collected from each patient during dosage, a few ml. of toluene being used as a preservative. After a suitable period for exogenous steroid clearance an equivalent number of full 24 hr. urine specimens were collected as a control, while no therapy was being given to the patient. In the case of patient B, two such control samples were obtained. Volume records were kept and it is believed that full 24 hr. specimens of urine were in fact collected. Each daily sample was worked up immediately it was received or was kept in the refrigerator and worked up as soon as was possible.

The urine, whether sample or control, was hydrolysed by boiling for 10 min. in N. acid (HCl). The hydrolysate was cooled rapidly and shaken with ether. The combined ether extracts were washed with N.NaOH and then distilled water and evaporated to dryness. The various daily extracts from the same patient were bulked at this stage. The total neutral ether soluble extract was chromatographed on aluminium oxide and the total acetone eluate collected. This total acetone eluate was refluxed with a small volume of dry benzene, allowed to stand overnight and the crystals filtered off. The solid

was/

was rebulked in the original flask while the filtrate was either taken to dryness and re-crystallized from methanol or rechromatographed through aluminium oxide using mixed solvents of increasing polarity as eluants. All eluted fractions containing crystalline material were bulked and recrystallized from methanol.

The solid material left from the first benzene extraction was re-extracted with a small quantity of benzene and allowed to stand overnight before filtering. The filtrate was treated in the same way as the first such filtrate.

Care was taken to ensure that the sample and control urines from the same patients received, as far as was possible, precisely the same treatment.

Allopregnane-3α,20α-diol was isolated from each of urines collected during progesterone dosage. The extracts from patients A and C, the females, contained large amounts of highly coloured gums which could not readily be removed by chromatography and which tended to keep the steroids in solution. This difficulty, although overcome, has lessened the possibility of the yields being quantitative. The extracts from the urine of patient B, the male, were singularly free from oils and gums and consequently the allopregnane-3α,20α-diol was very/

very easily obtained which makes the yield more significant.

The working up of the control urine from patient A presented some difficulty as there was but little crystalline diol material to mark the position of any allopregnane-3α,20α-diol which may have been present. Consequently each fraction of the eluate from the second chromatograph which showed signs of containing crystalline material was leached with chilled acetone. The crystalline residue was identified as pregnane-3α,20α-diol while the solid from the acetone leachings yielded only one crystalline fraction on fractional sublimation under reduced pressure. This fraction was also identified as pregnane-3α,20α-diol, but no allopregnane-3α,20α-diol was found.

The control urine from patient B presented no great difficulty as very small amounts of oily pigment were present. The isolation of a few mg. of pregnane-3α,20α-diol indicated that the two days allowed for the clearance of exogenous steroid had probably been insufficient. Although this overlap was rather unfortunate from a theoretical viewpoint, practically the presence of these few mg. of pregnane-3α-20α-diol provided a marker for the position of

any/

any allopregnane-3α,20α-diol,had it been present, and so made the work both simpler and more accurate. The presence of this small amount of pregnane-3α-20α-diol in the control caused the realization that a further control should be performed using urine extracts containing an amount of pregnane-3α-20α-diol equivalent to that found in the sample urines.

Accordingly, a further 14 day collection of urine was obtained from patient B while no therapy was being administered and the neutral ether soluble portion was obtained from the acid hydrolysed urine. Pregnane-3α,20α-diol (300 mg.), which was demonstrably free from allopregnane-3α,20α-diol, was added and the mixture homogenized by solution in ethanol and subsequent evaporation to dryness. This control was worked up using the methods and quantities of solvent used for the sample, but no allopregnane-3α,20α-diol could be detected.

The control urine from patient C provided the same difficulties as that from patient A, but they were more readily overcome as some pregnane-3α, 20α-diol, which was present because of the insufficiency of the two day clearance period, provided precise knowledge of the whereabouts of any allo-

pregnane-3α,20α-diol. Only a few mg. of pregnane-3α,20α-diol were isolated and no indication of the presence of allopregnane-3α,20α-diol was found.

Experimental

Total neutral ether-soluble fraction:

Each individual urine sample was either hydrolysed and worked up immediately or was stored in the refrigerator and worked up as soon as possible. At no time did more than two days elapse between receipt of sample and its hydrolysis.

The toluene was removed from each urine sample by means of a large separating funnel. Urine volume was noted and it was transferred to a large conical flask. The urine was brought to the boil, 10% (v/v) of concentrated HCl was added and the mixture boiled for 10 min. The hydrolysate was cooled rapidly and extracted 3 x 1/3 vol. of peroxide-free ether. The combined ether extract was washed 3 x 1/3 vol. of N.NaOH and then 3 x 1/3 vol. of distilled water. The ether was distilled off leaving the neutral ether-soluble portion. The various daily neutral ether-soluble portions from a particular patient were bulked giving the total neutral ether-soluble fraction.

Total Acetone Eluate:

The total neutral ether-soluble fraction from any one patient was dissolved in dry benzene and absorbed from it on to a column of slurry-packed Grade II (Brockmann, 1941) Al_2O_3. The weight of Al_2O_3 used was 30 x the fraction weight. The column was eluted with benzene and then dry ether until no more material came off. Acetone was then used to elute the diol mixture and was passed until no further solid was being eluted. Evaporation of the acetone gave the total acetone eluate.

Patient A. A normal postmenopausal woman. Age prevented much travelling and urine supplies were regular and reliable.

The patient received 50 mg. progesterone per day intramuscularly for 22 days. Complete 24 hr. specimens of urine were collected during dosage period. The author received 3/5 of each 24 hr. specimen, the remaining 2/5 were used by another worker in the Department. The author therefore received the equivalent of 13.2 days' urine with a progesterone dose of 660 mg.

Total neutral ether-soluble fraction was a dark red gum which weighed 1.50 g.

Total /

Total acetone eluate was a dark purple crystalline solid which weighed 180 mg.

The total acetone eluate was refluxed for 1 hr. with 25 ml. dry benzene and allowed to stand overnight. The solid was filtered off at the pump and rebulked with the acetone eluate. The filtrate was evaporated to dryness, giving a deep red gum which weighed 117 mg. (fraction B.S.I).

B.S.I was allowed to stand in 2 ml. methanol, in which it was readily soluble, for several days. The crystals which had separated after 7 days were filtered off, washed with 0.5 ml. ice-cold methanol, and recrystallized from the minimum volume of methanol.

The yield was 1.9 mg. of fairly thick colourless rods which melted at 240-244°C. after a preliminary softening at 238°C. A mixture with authentic allopregnane-3α,20α-diol (M.P. 242°C.) melted at 241-243°C. and a mixture with authentic pregnane-3α,20α-diol (M.P. 236°C.) melted at 213-215°C.

The remainder of the yield (1.7 mg.) was acetylated by heating it at 100°C. for 2 hr. with 0.2 ml. pyridine and 0.1 ml. acetic anhydride. The diacetate was isolated by adding a few ml. of water and filtering without suction. The solid was dried

over/

over solid NaOH in vacuo overnight and then leached through the filter paper with a few ml. of hot methanol. The methanol was evaporated off, leaving 1.4 mg. of crude diacetate which melted at 134-140°C. A mixture with authentic allopregnane-3α,20α-diol diacetate (M.P. 137-139°C.) melted at 136-137°C.

The remainder of the total acetone eluate (63 mg.) which was nearly colourless was refluxed with 25 ml. dry benzene for 1 hr. and allowed to stand overnight. The crystal crop was filtered off at the pump and returned to the original bulk. The filtrate was evaporated to dryness, dissolved in the minimum volume of methanol and allowed to stand for a week in this constant volume. The crystals which had formed were obviously the feathery needles typical of pregnane-3α,20α-diol, but they were filtered at the pump and after drying melted at 235-236°C. A mixture with authentic pregnane-3α,20α-diol (M.P. 236-237°C.) melted at 235-236°C.

Yields: 1.9 mg. of allopregnane-3α,20α-diol.
approx. 63 mg. of pregnane-3α-20α-diol.

Approximate ratio of pregnane-3α,20α-diol: allopregnane-3α-20α-diol is 30:1.

Patient/

Patient A: Control.

One week after the last dose of progesterone 17 full 24 hr. specimens of urine were collected.

The total neutral ether-soluble fraction was a dark red oil weighing 1.70 g.

The total acetone eluate was a purple oil weighing 105 mg.

The total acetone eluate was refluxed for 1 hr. with 25 ml. dry benzene and allowed to stand overnight. No crystals were deposited so the material was absorbed, from the benzene, on to a 0.5 g. column of Grade II (Brockmann, 1941) Al_2O_3. The chromatograph was developed with 100 ml. benzene and the material was eluted with mixed solvents of gradually increasing polarity. The solvents used were ether-benzene mixture, ether, acetone-ether mixtures and acetone in that order. Each eluted fraction (15 ml.) was evaporated to dryness and allowed to stand overnight in a drop or two of acetone. (The evaporation of the acetone in this treatment causes oily material to creep up the sides of the flask and makes any crystalline material easily seen). Crystalline material was found in fraction 16 which was eluted by the first 15 ml.of

50% /

50% (v/v) acetone in ether; the previous eluant had been 5% (v/v) acetone in ether. Crystalline material could not be found in any other eluted fraction.

Fraction 16, a brown oily solid weighing 4 mg., was leached with 0.5 ml. of chilled acetone which left a few colourless needle shaped crystals which when dry melted at 231-234°C. This when mixed with authentic allopregnane-3α,20α-diol (M.P. 243°C.) melted at 215-228°C. and when mixed with authentic pregnane-3α,20α-diol (M.P. 236-237°C.) melted at 231-235°C.

The acetone leachings were submitted to micro-sublimation; fractionation was obtained by altering the position of the sublimation tube in the hot copper block. Some light oils distilled at fairly low temperatures, but the crystalline fraction sublimed at 145°C. at a pressure of 8×10^{-5} mm. of mercury. This fraction (approximately 0.5 mg.) which had no definite crystalline shape, melted at 232-238°C. A mixture with authentic pregnane-3α,20α-diol (M.P. 236-237°C.) melted at 230-235°C. and a mixture with authentic allopregnane-3α,20α-diol (M.P. 243-244°C.) melted at 215-230°C.

Patient B.

A male, with chronic rheumatoid arthritis.

Sample. A daily dose of 100 mg. of progesterone was administered intramuscularly for 14 days. Complete 24 hr. urine specimens were collected during dosage period.

The total neutral ether-soluble fraction was a brown crystalline solid which weighed 1.52 g.

The total acetone eluate was refluxed for 1 hr. with 25 ml. of dry benzene and allowed to stand overnight. The crystals were filtered off and returned to the original flask. The filtrate was run through a 15 g. column of wet-packed Grade II (Brockmann, 1941) Al_2O_3, the chromatograph was developed with 400 ml. of benzene and the material was eluted with mixed solvents of gradually increasing polarity. Each eluted fraction (25 ml.) was taken to dryness and treated with a few drops of acetone to detect crystals. Crystalline material was found in each of six consecutive fractions; the first was eluted with 5% (v/v) acetone in ether and the sixth with pure acetone.

All/

All six fractions were bulked and dissolved in the minimum amount of methanol. After a few days in the refrigerator a crop of glistening rods appeared and were filtered off. Yield 4.5 mg. of rod-like crystals which melted at 243-244°C. A mixture with authentic allopregnane-3α,20α-diol (M.P. 243-244°C.) melted at 243-244°C. and a mixture with pregnane-3α-20α-diol (M.P. 236-237°C.) melted at 215-218°C. Careful working over of the methanol mother liquors did not yield any more allopregnane-3α,20α-diol.

A second extraction of the remainder of the total acetone eluate with 25 ml. of benzene and treatment of the benzene soluble portion by the methods given above did not yield any more allopregnane-3α-20α-diol.

The remainder of the total acetone eluate (120 mg.) after the 50 ml. of benzene-soluble material had been removed, although still light brown in colour, melted at 234-235°C. A mixture with authentic pregnane-3α-20α-diol (M.P. 236-237°C.) melted at 234-236°C.

Yield: 4.5 mg. of allopregnane-3α-20α-diol.

Approximately 120 mg. of pregnane-3α,20α-diol.

The/

The approximate ratio of pregnane-3α,20α-diol: allopregnane-3α,20α-diol was 30:1.

Patient B. Control I.

Starting two days after the last dose of progesterone 14 complete 24 hr. urine specimens were collected.

The total neutral ether-soluble fraction was a deep red gum which weighed 1.03 g.

The total acetone eluate was a red gum which weighed 215 mg.

The total acetone eluate was completely soluble in 25 ml. of dry benzene in the cold. This benzene solution was therefore run through a 15 g. column of wet-packed Grade II (Brockmann, 1941) Al_2O_3, the chromatograph was developed by 300 ml. benzene and the material was eluted with mixed solvents. Fractions 6, 7 and 8, successive 25 ml. portions of the 50% (v/v) acetone in ether eluate, were the only ones which developed crystals when treated with a drop or two of acetone.

Fractions 6, 7 and 8 when leached rapidly with 0.5 ml. of cold acetone each gave fairly clean crystalline material which melted over the range 190-225°C. These solids were bulked and recrystallized from/

from the minimum volume of methanol to yield needle crystals which melted at 234-235°C. A mixture with authentic pregnane-3α,20α-diol (M.P. 236-237°C.) melted at 235-236°C. The methanol mother liquors when taken to dryness and sublimed gave 2.5 mg. of crystalline material which came over at 130-160°C. under a pressure of 5×10^{-4} mm. of mercury. This was the only crystalline fraction. This material melted at 227-233°C. and after recrystallization from 2 ml. of acetone, gave platelets which melted at 234-236°C. and which did not depress the melting-point of pure pregnane-3α-20α-diol.

The acetone leaching liquors were bulked, taken to dryness and sublimed under high vacuum. Fractionation of the subliming material was obtained by moving the sublimation tube in the hot copper block to present a fresh surface to the material subliming at any particular temperature. No crystalline material sublimed between 50° and 165°C. at a pressure of 1×10^{-4} mm. of mercury.

Purification of a stock of pregnane-3α,20α-diol.

A stock of pregnane-3α,20α-diol (5 g.) was refluxed for 1 hr. with 100 ml. of dry benzene and allowed to stand overnight. The solid was filtered off/

off, dried and stored as 'purified' pregnane-3α,20α-diol. The benzene filtrate was taken to dryness and recrystallized from a small volume of methanol. After standing at constant volume for several days the crystal crop was isolated by filtration and found to melt at 236-237°C. A mixture with authentic pregnane-3α,20α-diol (M.P. 236-237°C.) melted at 236-237°C.

The 'purified' pregnane-3α-20α-diol was therefore free from allopregnane-3α,20α-diol.

Control II. A further 14 day collection of urine was made by patient B some two months after the progesterone administration had stopped. The patient received no therapy during this 14 day period.

The total neutral ether-soluble fraction was a deep red gum which weighed 1.04 g. To this neutral fraction 300 mg. of 'purified' pregnane-3α,20α-diol was added, the mixture dissolved in ethanol and then evaporated to dryness.

The total acetone eluate was then obtained; it was a light brown crystalline solid which weighed 790 mg.

The total acetone eluate was refluxed with 25 ml. of dry benzene for 2 hr. and allowed to stand/

stand overnight. The solid was filtered off at the pump and returned to the original flask. The filtrate was run through a 5 g. column of Grade II (Brockmann, 1941) Al_2O_3. The chromatograph was developed by 200 ml. of benzene and the material was eluted by 25 ml. portions of mixed solvents of increasing polarity. Crystalline material was detected in fractions 12-15 inclusive; these had been eluted by acetone/ether mixtures.

Fractions 12, 13, 14 and 15 were bulked and dissolved in the minimum volume of methanol and left in the refrigerator for a week. The crystalline material was filtered off and when dry it melted at 230-234°C. A mixture with authentic pregnane-3α,20α-diol (M.P. 236-237°C.) melted at 233-235°C. The methanol mother liquors gave another crop of pregnane-3α-20α-diol when the volume was reduced.

Patient C. A postmenopausal woman with rheumatoid arthritis.

Sample. The patient received a daily dose of 100 mg. of progesterone intramuscularly for 14 days. Complete 24 hr. urine specimens were obtained for each of the 14 days.

The total neutral ether-soluble fraction

was/

was a dark red oil which weighed 865 mg.

The total acetone eluate was a purple crystalline solid which weighed 316 mg.

The total acetone eluate was refluxed with 50 ml. of dry benzene for 2 hr. and allowed to stand overnight. The solid was filtered off and the filtrate was run through a 15 g. column of Grade II (Brockmann, 1941) Al_2O_3. The chromatograph was developed by 300 ml. benzene and the material was eluted with mixed solvents. Crystalline material was eluted by 5% (v/v) acetone in ether and continued to be eluted until pure acetone brought off the end of the band.

All the fractions which contained crystalline material were bulked and stood in 2 ml. of methanol for several days. The crystalline crop was filtered off and recrystallized from the minimum volume of methanol. The yield was 2.3 mg. of fine rod-like crystals which melted at 242-244°C. A mixture with authentic allopregnane-3α,20α-diol (M.P. 243-244°C.) melted at 242-243°C.

The mother liquors were not examined as they contained a large amount of purple gum.

Patient/

Patient C. Control. Two days after the last dose of progesterone urine collection was restarted. Complete 24 hr. specimens were obtained for 14 days.

Total neutral ether-soluble fraction was a dark red oil which weighed 335 mg.

Total acetone eluate was a purple oil which weighed 126 mg.

The total acetone eluate was readily soluble in 25 ml. benzene and so was rechromatographed through a 5 g. column of Grade II (Brockmann, 1941) Al_2O_3. Elution with mixed solvents gave several consecutive fractions containing some crystalline material. All such fractions were bulked. Too much purple oil was present to permit direct crystallization from methanol, so the material was sublimed. The fraction which sublimed at 100-160°C. at 5×10^{-4} mm. of mercury was leached with 0.2 ml. of chilled acetone while still in the sublimation tube and the solids left were washed into a small conical flask with hot methanol. On reduction of the methanol volume to 1.5 ml.,a good crystalline crop was obtained after a few days. This material melted at 233-236°C. when dry and on admixture with authentic pregnane-3α,20α-diol (M.P. 236-237°C.) it melted at 235-236°C.

Discussion .

The isolation of allopregnane-3α,20α-diol in increased quantities from the urine of human patients after intramuscular administration of progesterone proves that progesterone is a metabolic precursor of allopregnane-3α,20α-diol. The amount isolated from the urines of patients A and B, where the experimental work was fairly straightforward, indicates that about 0.3% of the administered progesterone was transformed into urinary allopregnane-3α,20α-diol.

It is interesting to note that the ratio pregnane-3α,20α-diol isolated: allopregnane-3α,20α-diol isolated, determined for the urines from patients A and B, was of the order of 30:1, which is not significantly different to the ratio found in human late pregnancy urine (Part II). This suggests that not only is progesterone a metabolic precursor of allopregnane-3α,20α-diol but that most, if not all, of the urinary allopregnane-3α,20α-diol present during pregnancy arises from progesterone.

Recently it has been shown that patients suffering from rheumatoid arthritis excrete an abnormally large proportion of intramuscularly administered/

administered progesterone as urinary pregnanediol (Sommerville, Marrian, Duthie and Sinclair, 1950). It is felt, however, that although this abnormality would occur in patients B and C, both rheumatoid arthritics, the close relationship of the results from normal patient A and those from patient B enables the deductions drawn to remain valid.

Summary.

1. Allopregnane-3α,20α-diol has been isolated from the urine of human patients, who were shown to have no detectable amount of endogenous allopregnane-3α,20α-diol, after intramuscular administration of progesterone.

2. The amount of allopregnane-3α,20α-diol accounted for about 0.3% of the administered progesterone.

3. The ratio (30:1) of pregnane-3α,20α-diol: allopregnane-3α,20α-diol arising from administered progesterone is not significantly different from that found in human late pregnancy urine.

4. It is suggested that most, if not all, of the allo-pregnane-3α,20α-diol present in human late pregnancy urine arises from progesterone.

administered progesterone as urinary pregnanediol (Sommerville, Marrian, Duthie and Sinclair, 1950). It is felt, however, that although this abnormality would occur in patients B and C, both rheumatoid arthritics, the close relationship of the results from normal patient A and those from patient B enables the deductions drawn to remain valid.

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General Discussion.

The initial isolation of the allopregnane-3α,20α-diol from the 95% (v/v) acetone mother liquors from the crystallization of 'sodium pregnanediol glucuronidate', which was essentially a chance isolation, requires no comment. The richness of, and the ease of isolation from, this source was however both interesting and instructive. The identification of the allopregnane-3α,20α-diol was quite straightforward (Part I).

The development of the direct method of isolation arose from the qualitative observations obtained during the initial isolation reinforced by the solubility data determined in Part II. Use of the method under controlled conditions has provided a new figure for both the relative quantities of pregnane-3α,20α-diol and allopregnane-3α,20α-diol isolable from, and for the absolute concentration of allopregnane-3α,20α-diol in, human late pregnancy urine. While it is felt that the absolute concentration proposed for allopregnane-3α,20α-diol (0.6-0.8 mg./l.) is of the correct order, the possible 30% loss indicated by the control experiments plus any loss of pregnane-3α,20α-diol which may have occurred during the practical work makes the ratio of 30:1 an/

an isolation figure rather than a representation of the relative amounts actually present in the urine. The difference between the figure now proposed for the concentration of allopregnane-3α,20α-diol in human late pregnancy urine (0.6-0.8 mg./l.) and the accepted one (6.6 mg./l.) is surprisingly large in spite of the suspicion with which the previous figure had been viewed (Introduction, pp. 11-16).

The removal of allopregnane-3α,20α-diol, or its conjugate, from 'sodium pregnanediol glucuronidate' by repeated acetone precipitations (Part III) was of course strongly indicated by the source from which the original isolation was made. The fact that more than two acetone crystallizations of the glucuronidate are required for this removal, and the isolation of the trioxygenated steroid from the hydrolysis products of rigorously purified 'sodium pregnanediol glucuronidate', indicates that this complex may well be a grosser mixture than has been previously supposed.

The proof that progesterone is a precursor of allopregnane-3α,20α-diol merely confirms a widely held hypothesis, but the isolation of pregnane-3α,20α-diol and allopregnane-3α,20α-diol in the same ratio after progesterone dosage as from human pregnancy urine/

urine does suggest that most if not all of the allopregnane-3α,20α-diol in such a urine arises from progesterone. This casts some doubt on, but does not deny, the hypothesis of a possible adrenal precursor suggested by the common occurrence of the allo configuration in saturated adrenal steroids. This work also provides the first proof that the metabolic reduction of progesterone is not stereochemically specific.

The suggestion that most of the urinary allopregnane-3α,20α-diol arises from progesterone requires of course further work for confirmation or denial. A necessary corollary of the suggestion is that the excretion of allopregnane-3α,20α-diol during pregnancy should rise in parallel with the rising progesterone output as indicated by the rising urinary excretion of pregnane-3α,20α-diol. While the method devised and used here will certainly not permit any fine distinction between the amounts of allopregnane-3α,20α-diol excreted in successive weeks, it should most certainly provide a means of assay of the amounts present in,say,the second, fourth, sixth and eighth month of a human pregnancy. Such a study, if a suitably cooperative patient or patients could be obtained, should be quite straightforward/

forward. The only obvious difficulty which suggests itself is the removal of the extra large amounts of gums and waxes relative to the allopregnane-3α,20α-diol which would be present in the urine extracts from these early pregnancies.

It is perhaps pertinant to note here that the urinary excretion of allopregnane-3α-ol-20-one, which one would expect on purely chemical grounds to occur in the progesterone-allopregnane-3α,20α-diol metabolic pathway, has been followed throughout a normal pregnancy (Dobriner, Lieberman, Rhoads and Taylor, 1948). The quantitative measurements, obtained by infra-red spectrography, are rather surprising. They indicate that the non-pregnant female does not excrete a detectable amount of allopregnane-3α-ol-20-one but that by the third month of pregnancy the excretion has risen to some 2 mg./24 hr. After the third month there is a rapid fall in the excretion level to about 1 mg./24 hr.; it then gradually rises to a level of about 2.5 mg./24 hr. at term, when it returns rapidly to zero. The existence of this high level of excretion at the third month is rather surprising if true, but as the authors themselves interpret their results as follows: 'Allopregnanolone seems to be excreted at/

at a relatively constant level of 1-2 mg./24 hr. throughout pregnancy', it would appear that they accept that their methods have rather a large probable error.

There are two points worth noting which arise from this work. Firstly, the urinary excretion of this allopregnane-3α-ol-20-one just before term is of the same order as that found here for allopregnane-3α,20α-diol during late pregnancy. Secondly, if an equilibrium exists in the body between this allopregnane-3α-ol-20-one and allopregnane-3α,20α-diol, in a similar fashion to that occurring between pregnane-3α-ol-20-one and pregnane-3α,20α-diol, then it would be expected that the excretion of allopregnane-3α,20α-diol would rise slowly as pregnancy progressed in parallel with the slightly rising output of allopregnane-3α-ol-20-one indicated in the graph due to Dobriner et al. It appears, therefore, that a following of the excretion of allopregnane-3α,20α-diol throughout a pregnancy would hold considerable interest.

It is disappointing that none of the work reported in this thesis throws any light on the mode of occurrence of the allopregnane-3α,20α-diol in human/

human pregnancy urine. The results obtained herein are quite consistent with it occurring free, as the sulphate, the glucuronide or as some other water soluble derivative. Although certain preliminary experiments involving direct extraction of fresh samples of human late pregnancy urine with ether have shown quite clearly that it is not excreted in the free state a method for a further attack on this problem has not been devised. Investigations of several other batches of mother liquor solids from 'sodium pregnanediol glucuronidate' preparations by extraction and leaching with both polar and non-polar solvents (including acetone precipitation of the material from concentrated solutions and ethanolic crystallization and leaching) have not shown any sign of even partial enrichment of the allopregnane-3α,20α-diol conjugate as judged by the examination of the various hydrolysis products. This is another problem to be borne in mind for future work.

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extractions of the original material, from methanol yielded 52.5 mg. of allopregnane-3α,20α-diol. The mother liquors from all crystallizations were carefully worked over to yield the maximum amount of allopregnane-3α,20α-diol.

This careful isolation of allopregnane-3α-20α-diol yielded 0.54 mg. from each litre of human late pregnancy urine.

The benzene insoluble material which weighed 1.70 g., was shown to be fairly pure pregnane-3α,20α-diol; this indicates that the relative amounts of pregnane-3α,20α-diol to allopregnane-3α,20α-diol isolable from human late pregnancy urine is 17 to 0.54, i.e. approximately 30:1.

Experimental.

The solubility of pregnane-3α,20α-diol and allopregnane-3α,20α-diol in various solvents.

The pregnane-3α,20α-diol used was recrystallized from ethanol until it had a constant melting-point of 236.5-237°C.

The allopregnane-3α,20α-diol used was recrystallized from methanol until it had a constant melting-point of 244.5-245°C.

The apparatus used consisted of a small flask and/